

LEAF ULTRASTRUCTURAL
STUDIES OF *Avicennia marina* IN
RESPONSE TO SALINITY
UNDER NATURAL
CONDITIONS

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ABSTRACT

In Richards Bay Harbour, the mangrove *Avicennia marina* exhibits a distinct natural productivity gradient. The fringe site, which is regularly inundated twice daily by tides, supports luxuriant adult *A. marina* trees that are 6-10 m tall and which form a dense, well-developed canopy. The landward site which is only inundated during high spring tides, supports diminutive or dwarf *A. marina* that are less than 1.5 m in height. In this study we compared leaves from fringe and dwarf sites with respect to morphology, ultrastructure and ecophysiology.

Alterations in leaf morphology, ultrastructure and physiology of *A. marina* were compared at the fringe site (35 ‰) and dwarf site (60 ‰) using morphometric measurements, light (LM), transmission (TEM) and scanning microscopy (SEM). SEM and light microscopy revealed that multicellular salt glands were located on the thick, cutinised adaxial surface from leaves of both sites. The glands appeared to be scattered and protruding from individual crypts in fringe mangrove leaves whilst they appeared sunken and occluded by cuticular material in dwarf mangrove leaves. The salt glands on the abaxial surface were not sunken but obscured by the indumentum of peltate trichomes. Ultrastructural changes observed in dwarf mangrove leaves were associated with cuticle, cell walls, chloroplasts, mitochondria of mesophyll tissue and salt glands. Fringe mangrove leaves had chloroplasts with typical well-developed grana and stroma. Ultrastructural changes of chloroplasts were evident in dwarf mangrove leaves and included swelling and separation of thylakoids, disintegration of granal stacking and integranal lamellae, as well as loss of the integrity of the chloroplast envelope. Multivesicular structures were commonly found in vacuoles and associated with chloroplasts and mitochondria in both leaf types. In fringe mangrove leaves, mitochondria appeared spherical to tubular with a relatively smooth outer membrane and a highly convoluted inner membrane. Swelling and vacuolation of mitochondrial membranes, cristae and mitochondrial clustering in the cytoplasm around the chloroplasts were evident in dwarf mangrove leaves. Extensive lipid accumulation in the form of large, dense plastoglobuli occurred in the chloroplasts of dwarf mangrove leaves.

There were characteristic differences in salt gland morphology of fringe and dwarf mangrove leaves, namely in the cell walls, vacuoles, and vesicle formation. In salt glands of dwarf mangrove leaves, a distinct withdrawal of the cytoplasm from the cell wall was observed. This feature was not observed in salt glands of fringe mangrove leaves. Numerous large vacuoles were observed in the secretory cells of glands of dwarf mangrove leaves compared to those of fringe plants. Multivesicular structures, vesicles and mitochondria were common features in both leaf types.

III

Physiological studies involved a comparison of osmotic and ionic relations as well as whole plant responses in fringe and dwarf mangrove leaves. Relative leaf water content decreased by 7.8 % and specific leaf area by 17 % in dwarf compared to those of fringe mangroves. Dwarf mangrove leaves were 27.6 % thicker and leaf cuticle thickness 37.4 % higher than those from fringe mangroves. Fringe mangrove leaves displayed higher total chlorophyll contents by 27 %, with chlorophylls a and b being 22 % and 39.6 % higher, respectively than those of dwarf mangroves. Salt gland frequencies were higher in the apex, mid-lamina and base of fringe than dwarf mangrove leaves by 36 %, 45 % and 51 %, respectively. The concentration of glycinebetaine, a compatible, N-containing osmolyte was significantly higher by 40 % in dwarf than in fringe mangrove leaves. Concentrations of proline were 27 % lower in dwarf than in fringe mangrove leaves. The predominant inorganic ion detected in mature leaves was Na⁺, which was 19 % higher in dwarf than fringe mangrove leaves. Phosphorus was an element that appeared deficient in dwarf mangrove leaves, being 50 % lower compared to fringe mangrove leaves.

The results of this investigation indicated that there were cytomorphological alterations as well as differences in physiological responses in leaves of *A. marina* at fringe and dwarf sites.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biological & Conservation Sciences, University of KwaZulu Natal, Westville, from February 2002 to December 2007, under the supervision of Professor Gonasageren Naidoo and co-supervised by Dr. Yougasphree Naidoo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Omitha Hiralal

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LIST OF ABBREVIATIONS

LM – Light Microscopy

SEM – Scanning Electron Microscopy

TEM – Transmission Electron Microscopy

b – globules of secretory substance

bs – ball of secretion

c – cuticle / cuticular material

Cl - chloroplast

CH – cuticular chamber

Co – collecting cell

Cp - cytoplasm

cr - cristae

Cr – crypt

CW – cell wall

E – epidermal cells / epidermis

Ec – extracytoplasmic space

g – granal stacks

H – hypodermal cells

h – concave heads

IAS – intercellular air space

LE – lower epidermis

M – mitochondria

MV – multivesicular structures

N – nucleus

nu - nucleolus

p - plastoglobuli

PM – plasma membrane

s – stomata

S – secretory cell

sg – starch grains

SG – salt gland

SM – spongy mesophyll

sSG – senescing salt gland

st – stoma

St – stalk

t – trichomes

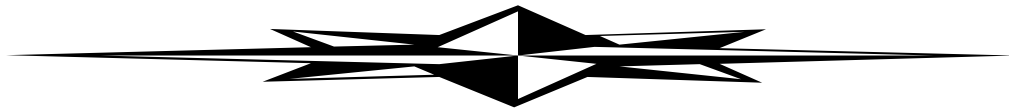
v – vesicles

Va – vacuole

VB – vascular bundle

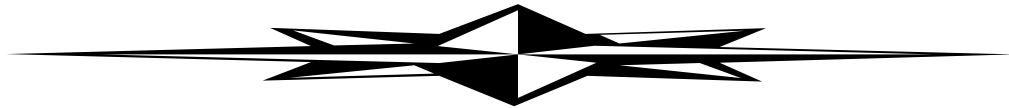
Author, Loren Eiseley (1971) wrote vividly about his encounter with a mangrove forest in the book *The Night Country*:

*“A world like that is not really natural
Parts of it are neither land nor sea and so
everything is moving from one element to
another
Nothing stays put where it began because
everything is constantly climbing in, or
climbing out, of its unstable environment.”*



Dedicated to

MY HUSBAND ~ Dilshad Singh



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CHAPTER 1

INTRODUCTION

Mangroves comprise a dynamic assemblage of plant communities that span the intertidal zones of the tropics and subtropics around the globe. Mangroves have developed adaptations unique to most other plants, enabling them to grow under fluctuating salinities and anaerobic conditions. Worldwide there are 69 recognised species of mangrove plants belonging to 20 families (<http://www.aims.gov.au/pages/reflib/fg-mangroves>).

1.1. Importance and ecological role of mangroves

Mangroves form one of the most biologically productive ecosystems (<http://www.nrca.org/>), which provide several important ecological and economic services including shoreline protection, productive commercial and sport fisheries and nutrient cycling as well as providing habitats for wildlife and nursery areas for associated fauna (<http://www.response.restoration.gov.za>; <http://www.nrca.org/>)

1.2. Protection

Mangroves provide shoreline protection from erosion by acting as a buffer zone, reducing impacts of strong winds, flooding by typhoons and tidal waves, minimising water pollution, and destruction of coral reefs (Mazda *et al.*, 1997; Mendoza & Alura, 2001). The tangled roots, pneumatophores, and trunks of the mangroves help assuage siltation by trapping sediment in neighbouring coastal habitats (<http://www.nrca.org/>). Mangrove ecosystems maintain water quality by trapping sediments in the root system therefore protecting other offshore waters of coastal ecosystems such as oyster beds, sea grasses and coral reefs from excessive sedimentation (Ewel *et al.*, 1998). Agrochemical and heavy metal pollutants from the water are consequently limited since these contaminants adhere to sediment particles. Mangroves also prevent downstream and coastal eutrophication through denitrification of organic and inorganic nutrients (Ewel *et al.*, 1998). Individuals, communities and the government can achieve the conservation and management of mangrove ecosystems by fulfilling the aims, goals, principles and policies as set in the Ramsar Convention on Wetlands of International Importance (<http://www.nrca.org/>). In the Eastern Coast of Samar Island in Philippines local government units have passed laws for

mangrove protection against destruction (Ewel *et al.*, 1998) and so have most countries including South Africa, e.g. Beachwood, St. Lucia and Richards Bay.

1.3. Damage

Damage to mangroves caused by biotic disturbances comprises competition, herbivory from insects and defoliation of mangrove leaves by caterpillars, predation of propagules by crabs and disease from fungi (<http://www.response.restoration.gov.za>). However, global mortality on mangrove forests includes periodic frosts, tropical storms and flooding that bring heavy sedimentation onto land (Jimenez & Lugo, 1985). Seawater intrusion onto land, as occurred in recent tsunami-affected areas have deposited large amounts of salts in coastlands. In addition, the timber industry, coastal development and aquaculture, i.e. anthropogenic activities cause major loss of mangrove ecosystems.

1.3.1. Negative impact of sea-level rise on ecosystems

Apart from the population growth crises that we may inevitably encounter in the coming years, we face a more drastic problem of global climatic change and the rise in sea level. All coastal ecosystems are affected by changes in sea levels (<http://www.response.restoration.gov.za>). Hydrological changes may, therefore, occur as the period and the level of flooding increases. The ability of mangrove ecosystems to adapt to such changes in hydrology will depend on the enormity of the change and their ability to either: (i) increase mangrove sediment elevation through vertical accretion, or (ii) migrate in a landward direction (<http://www.response.restoration.gov.za>).

A dynamic balance, in general, exists between mangrove sediment surface and sea level. Moderate sedimentation is valuable to mangroves as a source of nutrients and maintains the predicted intensity of eustatic sea level rise. However, sea level rise has accelerated and the rate of rise might be faster than the ability of mangrove forests to accumulate and stabilise sediments. The alternative is for mangroves to recede into previous uplands, but only if there is enough space to accommodate the mangroves at the new intertidal level. Nonetheless, local elevation gradients may make this regression impossible. With excessive sedimentation, the growth and productivity of mangroves is short-lived (<http://www.response.restoration.gov.za>). Ellison (1998) reported that complete burial of *Avicennia* roots to a depth of 10cm, would interrupt gas exchange, and kill root tissue and the trees.

A few millimeters per year in sea level rise may seem diminutive, yet in a case study documented by the Inter-governmental Panel on Climatic Change (IPCC) Working Group 3 concluded that a world-wide rise in sea-level would result in wetland and lowland inundations; shoreline erosions; increased salinisation of estuaries and coastal flooding, therefore impairing the quality of the water. In addition, tidal ranges in rivers and bays would change and sediment deposition of riverbeds would be altered (Ibe & Awosika, 1991).

1.3.2. Human exploitation of mangroves

There has been a considerable decline in the productivity of mangroves due to man's exploitation and over-utilisation of valuable resources during commercial, urban and industrial development. Deforestation is associated with firewood and timber harvesting, land reclamation for human establishment, cultivation of rice and sugarcane (<http://www.nrca.org/>), salt production, mariculture and pasture (Mendoza & Alura, 2001). Twilley (1998) reported that a residential project in Florida, USA, cleared out approximately 24% of mangrove cover; and in Ecuador, along the El Oro River, approximately 45-63% of the mangrove environment was eradicated due to the construction of a mariculture pond. Annihilation of mangrove areas has been reported in Thailand (55%) and Philippines (60%), (<http://www.response.restoration.gov.za>).

1.3.3. Pollution

Pollution of mangrove ecosystems caused by man includes thermal (hot water outflows), heavy metals, agrochemicals, nutrients (including sewage) and oil spills (<http://www.nrca.org/>). Mining and industrial wastes are the main sources of heavy metal pollution especially mercury, lead, zinc and copper (De Lacerda & Abrao, 1984; De Lacerda *et al.*, 1993). Heavy metals adhere to sediments and may decrease growth and respiration rates of mangroves and negatively affect fauna. In addition, pesticides and heavy metals may bioaccumulate in mangroves and concentrations of mercury, cadmium, and zinc are toxic to invertebrates, fish larvae and crab reproduction (<http://www.aims.gov.au/pages/reflib/fg-mangroves>). Although sewage disposal may enhance tree growth and productivity, excessive dumping of wastes may cause high algal growth, cover mangrove pneumatophores, reduce seedling growth and reduce oxygen exchange (Hogarth, 1999).

1.4. Need for conservation

The world population is presently over six billion and it is estimated to exceed eight billion by 2025. This exponential growth rate is expected to arise from developing countries (Sharma &

Goyal, 2003). The global food production will need to increase by 38% by 2025 and by 57% by 2050 (Wild, 2003) if food supply is maintained at its current production rates (Rengasamy, 2006). With the escalating sea-level rise, the existing ecological problems of coastal erosion, drought, flooding and deforestation are overwhelming. The need for conservation of our mangrove ecosystems is extremely important. The adverse effects of salinity stress and increased waterlogging caused by increasing sea level rise would consequently have devastating effects on our coastal ecosystems that cannot acclimatize to saline, waterlogged conditions.

1.5. Distribution of mangroves

Avicennia is a pantropical genus of 8 species and together with *Rhizophora* are the dominant plants of mangrove communities throughout the world, each genus having several closely related species in both the Atlantic East Pacific and the Indo West Pacific (MacNae, 1968; Tomlinson, 1986; Ellinson, 1991).

Mangroves are distributed within the tropical and subtropical zones of the world, occurring predominantly between 25°N and 25°S (Fig. 1.) (<http://www.response.restoration.gov.za>). *Avicennia marina* is broadly distributed both latitudinally and longitudinally, ranging along the south, east and west coasts of Africa, southern Asia and Australia (Little, 1983). On the Atlantic coast of America and Africa, where the sea surface temperature is closer to 27°C, mangroves like *Avicennia germinans* in the Western Hemisphere are less tolerant of cold than the species in the Eastern Hemisphere. Mangroves can endure air temperatures below 27°C, but have low tolerances for frost, which adversely affect their growth and productivity (Tomlinson, 1986).

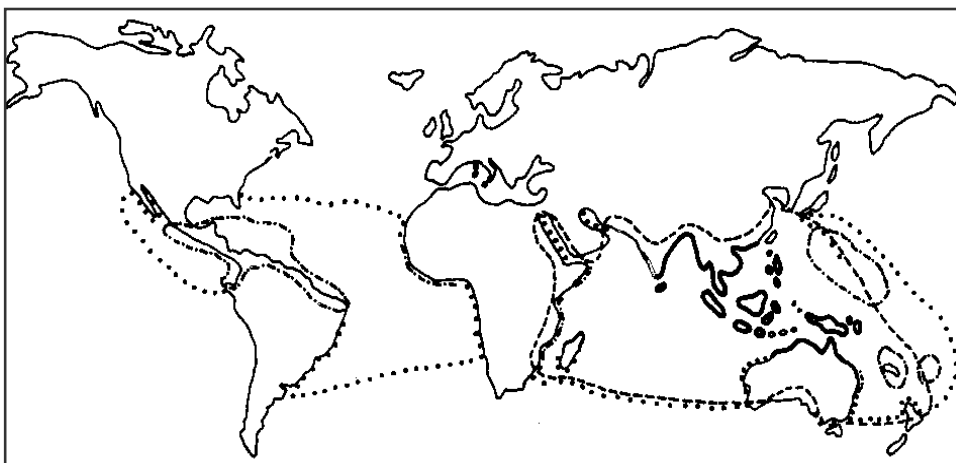


Fig. 1. General world distribution of mangroves (<http://www.aims.gov.au/pages/reflib/fg-mangroves>).

1.6. Distribution of *A. marina* in southern Africa

A. marina is a perennial C_3 , halophytic tree with luxuriant canopies that branch into numerous small branchlets (Fig. 2d). Other species of *Avicennia* include *A. alba* and *A. germinans*, however, *A. marina* is the only dominant species in southern Africa. It produces a tangled mass of horizontal roots that spread out far from the base of the trunk, and anchored well into the mud. Small, pencil-like roots called pneumatophores develop from these roots and project through the mud to facilitate their ventilation and absorption of oxygen (Fig. 2c,e).

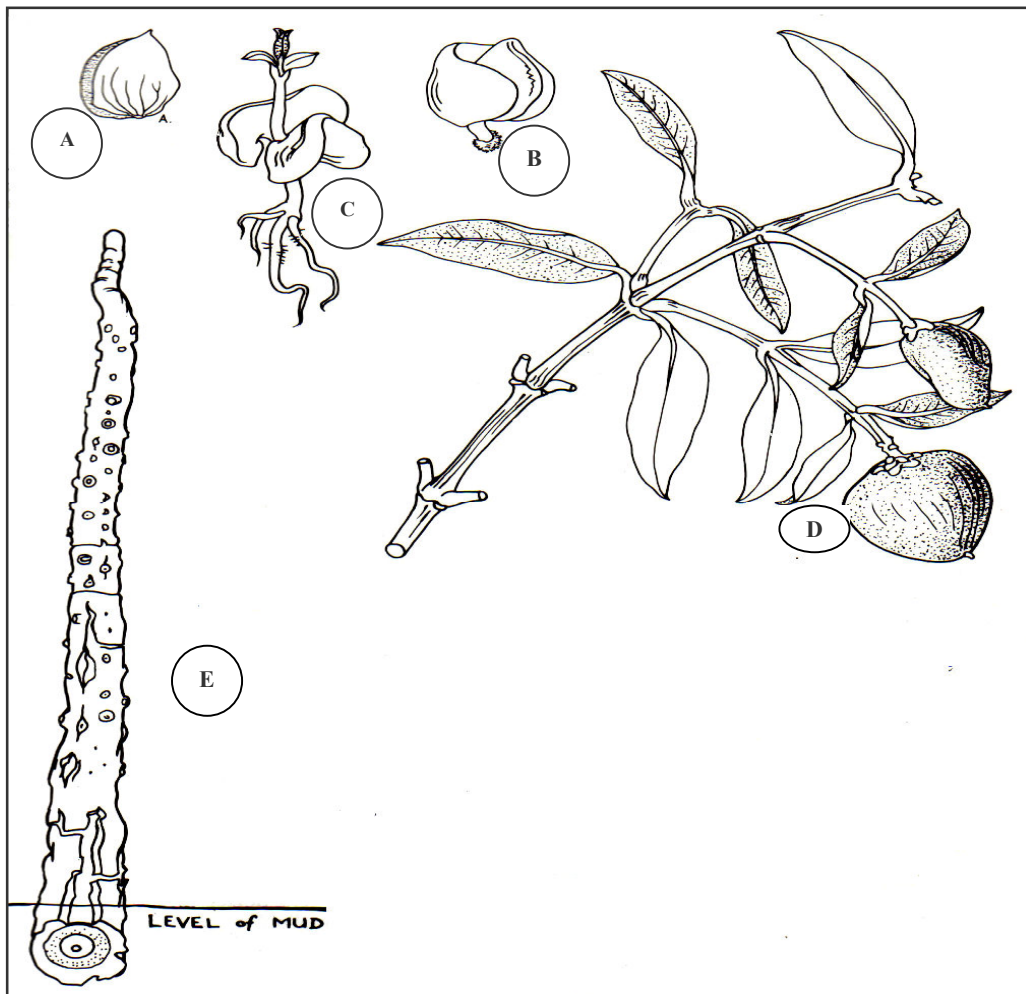


Fig. 2. *A. marina*. a. The cotyledons appear thin-skinned. b. The cotyledons are folded with the radicle appearing. c. The young plant. d. Leaves and fruit. e. Pneumatophore; drawn from Palmer & Pitman (1973).

The name *Avicennia* commemorates the Arabian philosopher of the first Millennium, Aba-bin-senna, who contributed to the development of European medicine (Tomlinson, 1986). *Avicennia marina* belongs to the family Avicenniaceae (Tomlinson, 1986) and is widely distributed in mangrove forests along the coastal belt of the east coast of South Africa. Occurrence of *A. marina* has been reported from northern KwaZulu Natal to its southern limit near East London in the Eastern Cape (Fig. 3)

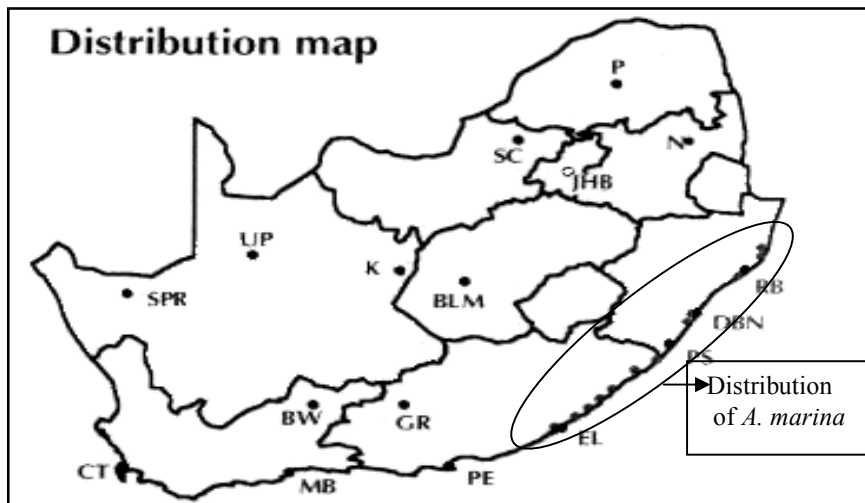


Fig. 3. Distribution map of *A. marina* along the coastal regions of southern Africa (after Palmer & Pitman, 1973).

1.7. Conclusion

Acknowledging and understanding the diverse nature of mangroves provide us with greater insight into the complexities of this remarkable form of wetland (Ewel *et al.*, 1998).

Although mangrove ecosystems are protected in southern Africa, potential threats still exist in most areas such as the Richards Bay harbour and Durban Bay. Intertidal vegetation, low-lying areas and nutritive-rich substrates can be lost due to increased salinisation, imbalances in the tidal range, drought, sedimentation, and coastal flooding. The incessant problems of pollution from ships (oil spills) and industry (heavy metal discharge, dust from coal operations, sulphide fumes) have been documented to have adverse effects on fauna (Hemens *et al.*, 1975) and flora of mangrove ecosystems (Naidoo & Chirkoot, 2004).

For this reason, extensive research and stringent management strategies (monitoring and evaluation) of mangroves need to be undertaken in order to ensure the sustained co-existence of plant and animal life as well as human developments hence minimising negative impacts on our coastal ecosystems.

1.8. Objectives

In Richard's Bay, *A. marina* is the dominant mangrove often occurring in monospecific stands along natural productivity gradients. The fringe site receives the brunt of tides twice daily and supports trees of 6-10 m whilst the landward site is only inundated during high spring tides and trees are dwarfed with statures less than 1.5 m. Soil salinity in the fringe mangrove site is about 35‰ while in the dwarf mangrove site, it is about 60 ‰ (Naidoo & Chirkoot, 2004). According to Huckle *et al.* (2000), the composition and distribution of plant communities along an elevational gradient is related to the ability to tolerate environmental conditions associated with tidal variation. Tidal inundation may vary in frequency and duration (Rozema *et al.* 1981; Huckle *et al.*, 2000), both of which decrease as the elevation of the gradient increases (Hubbard 1969 in Huckle *et al.*, 2000).

The study addresses the following questions:

1. Are there morphological differences between leaves of fringe and dwarf mangroves?
We tested the hypothesis that dwarf mangrove leaves have a lower specific leaf area, thicker leaves with thicker cuticles and greater salt gland frequency.
2. Are there ultrastructural differences between leaves of fringe and dwarf mangroves?
We tested the hypothesis that leaves of dwarf mangroves had reduced and damaged (smaller and disorganised) chloroplasts, mitochondria and other organelles and a larger salt gland frequency for more efficient salt secretion.
3. Are there physiological differences between leaves of fringe and dwarf mangroves in terms of concentration of inorganic and organic solutes?
We tested the hypothesis that dwarf mangrove leaves have higher concentrations of inorganic and organic ions for greater ionic and osmotic adjustment.

CHAPTER 2

LITERATURE REVIEW

Over the past five decades researchers have shown a significant interest in salinity and its effects on plant growth (Barrett-Lennard, 2003; Sharma & Goyal, 2003). There has been a vast literature on the effects of salinity on plants. According to the bibliography on plant responses to salinity published by USDA in 1978, 2357 references involving 1500 species and 50 crops were listed. The “BIOSIS” literature search for papers on salinity, salt resistance and vascular plants conducted by Flowers and Yeo (1995), revealed that in the 15 years (between 1978 and 1993) 4231 papers were published at a rate of 282 per annum. According to Sharma & Goyal (2003), their “BIOSIS 1993-2001” search indicated a decline in the number of published papers on salinity at a rate of 236 per annum with only 1890 additional papers. Despite the valuable accumulation of literature on salinity effects on plants, scientists worldwide are continually striving to develop ways to rectify the damage which is induced by salinisation, urbanization and industrialization on cultivated land.

Mangrove ecotypes

Mangroves comprise an assemblage of tropical trees and shrubs that form the dominant plant communities in tidal, saline wetlands (Paliyavuth *et al.*, 2004; Blasco *et al.*, 1996; Duke, 1992). Worldwide, they span an approximate area of 240 000km² of sheltered coastlines (Lugo *et al.*, 1990). Mangrove forests are categorised according to their patterns of distribution, structure, productivity, and biogeochemistry, and are controlled by an amalgamation of factors such as hydrology, soil characteristics and biological relations (<http://www.response.restoration.gov.za>).

The most common ecotypes consist of fringe, riverine, basin and scrub forests (Lugo & Snedaker, 1974; Twilley, 1998). Fringe mangroves border protected shorelines, canals and lagoons and receive the daily brunt of tides, which are often full-strength seawater (Lugo & Snedaker, 1974). In riverine mangroves, salinity is moderate, since the mangroves are flooded by nutrient-rich fresh and brackish river water as well as by tides (Twilley, 1998). Basin mangroves generally cover large areas behind fringe and riverine mangroves, with tides inundating the entire basin forest, harbouring stagnant, slow-flowing water.

Scrub or dwarf forests grow in areas of limited hydrology and result in conditions of high soil salinity at higher elevations, high evapotranspiration, low nutrient availability and accumulation of salts. The growth of scrub mangroves is impaired and stunted due to the stressful environmental factors listed above (Lugo & Snedaker, 1974; Ewel *et al.*, 1998; Twilley, 1998).

Mangrove adaptations to salinity

Salinity, site elevation and drainage affect the growth of mangroves across the tropics and subtropics (Bunt, 1996; Paliyavuth *et al.*, 2004). High salinity makes it more difficult for mangroves to extract water from the soil due to high evapotranspiration, especially in environments with restricted water flow (<http://www.response.restoration.gov.za>); and from soils that are waterlogged (Paliyavuth *et al.*, 2004). Mangroves alter their physiology and morphology to adapt to harsh environments thereby enabling successful colonization of intertidal zones (Khan & Duke, 2001).

Mangroves tolerate high soil salinities and therefore have low water potentials. This implies that the water potential of these plants need to be more negative than that of the soil solution to maintain water uptake and turgor (Ungar, 1991; Khan *et al.*, 2000). Smith *et al.* (1989) observed that during the dry season, due to the increase in salinity, the osmotic potential and xylem tension of *Avicennia germinans* became more negative to maintain turgor as compared to the rainy season.

The salt tolerance of mangroves increases during their growth and development (Sen *et al.*, 1982). However, accumulation of osmotically active substances in plant tissues can result in impaired metabolism attributable to high ion toxicity or electrolyte imbalance (Flowers, 1972; Greenway & Osmond, 1972). Aziz & Khan (2001) showed that *A. marina* accumulated high concentrations of Na⁺ and Cl⁻ ions during dry periods when the salt concentration was higher in the external medium. A favourable ionic balance is accomplished in mangrove shoots through various mechanisms, depending on the plant species. These mechanisms include salt secretion by glands, compartmentalisation of ions, succulence, abscission of salt-saturated organs, and salt exclusion by roots (Waisel, 1972).

According to Sen & Rajpurohit, (1982), succulence is one of the most common adaptive features of halophytes to reduce internal salt concentration. Strognov (1964) and Waisel (1972) suggested

that structural changes such as increase of succulence, changes in number and size of stomata, and thickening of cuticle can be attributed to salinity:

Generally, succulents lack the ability to secrete salts, but they “thwart” the rise of salt concentration by increasing their water content, thus becoming progressively succulent during their development (Zahran, 1982). Salt tolerance in the succulent salt marsh species, *Sarcocornia natalensis* was achieved by the accumulation of large amounts of inorganic solutes for osmoregulation; increased water flux and turgor-induced growth (Naidoo & Rughunanan, 1990).

Halophytes growing under high saline conditions have displayed the following adaptations (Parks *et al.*, 2002):

- (a) exclusion of Na^+ at the soil-root boundary and, therefore, from all tissues,
- (b) exclusion of Na^+ from the xylem and, therefore, from leaf tissues, thus preventing disruption of photosynthetic activities,
- (c) inclusion of Na^+ and synthesis of compatible solutes/osmoprotectants to maintain osmoregulation,
- (d) inclusion of Na^+ and its subsequent sequestration in vacuoles (Braun *et al.*, 1986; O’Leary, 1995; Blumwald *et al.*, 2000),
- (e) inclusion of Na^+ and its eventual elimination through secretion by leaves (Flowers *et al.*, 1977), and
- (f) abscission of leaves for eliminating excess salt (Kathiresan & Bingham, 2001; Cram *et al.*, 2002). Leaf drop involves losing both salt and biomass, however the salt concentration of the leaf sap is not reduced. Leaf fall is therefore not a “salt excretion mechanism, but is the point at which the leaf ceases to accumulate salt” (Cram *et al.*, 2002).

In the mangrove *A. marina*, salt resistance is based upon three different mechanisms: salt avoidance, salt tolerance and salt evasion (Waisel *et al.*, 1986).

According to Khan & Aziz (2001), *A. marina*, *Ceriops tagal* and *Rhizophora mucronata* showed optimal growth at 50% seawater. Na^+ and Cl^- ions increased with an increase in salinity, with the highest concentration of ions in *A. marina*. Optimal growth for mangroves has been variable ranging from 10 to 50% seawater (Naidoo, 1987; Karim & Karim, 1993; Khan & Aziz, 2001). An overall decline in plant growth, stomatal conductance and tissue water potential as well as an accumulation of inorganic ions are typical responses to increasing salinity (Ball & Farquahar, 1984a; Naidoo, 1987; Khan & Aziz, 2001).

Leaf senescence, dwarfism and inhibition of root growth, seed germination and leaf production are associated with typical conditions of extreme salt stress (Munns, 1993; Larcher, 1995; Werner & Finkelstein, 1995; Goldstein *et al.*, 1996; Neves-Piestun & Bernstein, 2001). Short-term salinity is considered to limit growth by inhibiting leaf expansion, whilst during long-term salinity, plants had a tendency to accumulate ions and lower carbon supply which could lead to premature senescence of mature leaves and a reduction in the photosynthetic area available to support further growth (Munns & Termaat, 1986; Cramer *et al.*, 1994). Stunted plants, therefore, have displayed less photosynthetic area, lower leaf production and thus lower survivability (Alpha *et al.*, 1996; Griffiths & Orians, 2003). Greenway & Munns (1980) suggested that growth reduction might be a consequence of salt effects on physiological processes, such as: dry matter allocation, ion and water relations. Wyn Jones (1985) and Blum (1988) suggested that plants exposed to saline conditions were under induced water and osmotic stresses, specific ion toxicities, and ionic imbalance.

Accumulation of osmoprotectants in response to stress in plants

Osmoregulation or osmotic adjustment has been demonstrated to be the maintenance of cell turgor by a sufficient increase in cell solutes to compensate for external osmotic stress (Zhang *et al.*, 1999). Hence, when plants experienced unfavourable environmental conditions associated with high levels of salt or drought, the plants acclimated by protecting themselves from these stresses (Bohnert *et al.*, 1995; Sakamoto & Murata, 2002; Wang & Showalter, 2004). When excessive salt accumulated in a cell, the consequences for living cell metabolism were deleterious as the excessive accumulated salts dehydrated the cytosol and subsequently denatured several essential metabolic enzymes (Datta *et al.*, 2003). Ideally, Na⁺ and Cl⁻ ions should be sequestered in the vacuole of the cell, and organic solutes, which are not inhibitors to metabolism (compatibles) should accumulate and be compartmentalised in the cytoplasm and within organelles (chloroplasts and mitochondria) to balance the osmotic pressure of the ions in the vacuole, thereby increasing salt tolerance (Apse *et al.*, 1999; Heldt, 1999; Hasegawa *et al.*, 2000; Sakamoto & Murata, 2002; Cuin *et al.*, 2003).

Compatible solutes were found to be small molecules that were water-soluble, and neutral with respect to perturbations in cellular functions, even in high concentrations (Yancey *et al.*, 1982). Plants responded to stress by accumulating a variety of osmoprotectants Naidu (1998) such as:

- (a) sugars and sugar alcohols (polyols) (Yancey *et al.*, 1982).
- (b) proline (Aspinall & Paleg, 1981) and its analogues (Naidu *et al.*, 1987), and

- (c) a number of quaternary ammonium compounds (betaines such as glycinebetaine, dimethylglycine) (Wyn Jones & Storey, 1981; Rhodes and Hanson, 1993). Plants accumulated these solutes as an adaptive mechanism to environmental stresses such as salinity (Hayashi *et al.*, 1997), water deficit (Monyo *et al.*, 1992; Zhang *et al.*, 1999) and temperature extremes (Yang *et al.*, 1996; Hayashi *et al.*, 1997).

Glycinebetaine (*N,N,N''*-trimethylglycine) was found to be a quaternary ammonium compound (Mäkelä *et al.*, 1998a) that was electrically neutral over a wide range of physiological pH values (Sakamoto & Murata, 2002). It was very soluble in water but included a non-polar hydrocarbon moiety that comprised of three methyl groups (Gorham, 1995; Sakamoto & Murata, 2002). The synthesis of glycinebetaine has been shown to involve a two-step oxidation of choline, catalysed by choline mono-oxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) in chloroplasts (Papageorgiou *et al.* 1991; Rhodes & Hanson, 1993, Rahman *et al.*, 2002).

Glycinebetaine has been a compatible organic solute in the osmotic adjustment of cytoplasmic compartments where it might have been shown to accumulate while inorganic ions were sequestered in the vacuole (Robinson and Jones, 1986; Matoh *et al.* 1987; Rhodes and Hanson, 1993). Glycinebetaine accumulated in large amounts to maintain osmotic balance/osmoregulation in different *Avicennia* species and to counteract the toxic effects of high Na⁺ and Cl⁻ concentrations (Popp & Albert, 1995, Takemura *et al.*, 2000). It was demonstrated that in *A. marina* leaves, glycinebetaine occupied approximately 5-10 % of cytoplasmic volume of the cell, which was sufficient to balance the total osmotic potential of the leaf, preventing cell dehydration, and excess inorganic ion accumulation (Wyn Jones & Storey, 1981; Downton, 1982; Ashishara *et al.*, 1997). Khan *et al.* (1998) reported that glycinebetaine served as a suitable osmoticum as its content increased with increasing NaCl in the halophytes, *Atriplex griffithii*, *Haloxylon recurvum* and *Halopyrum mucronatum*.

Beneficial effects of glycinebetaine and proline to halophytic plants

High concentrations of salts inhibited enzyme activity in both glycophytic and halophytic higher plants (Flowers *et al.*, 1977). In contrast, enzyme activity was unaffected by high concentrations of glycinebetaine. Stress-induced glycinebetaine accumulation was presumably adaptive (Khan *et al.*, 1998). It also served to 'protect' the O₂ evolving machinery of chloroplasts and to stabilise photosynthetic reactions, and membrane and protein functions when exposed to high NaCl

concentrations (Mamedow *et al.*, 1991; Murata *et al.*, 1992; Hanson *et al.*, 1995; Papageorgiou & Murata, 1995).

In vitro studies have shown that glycinebetaine enabled to protect the activities of enzymes, such as rubisco and malate dehydrogenase, from the inhibitory effects of concentrated NaCl or KCl (Pollard & Wyn Jones, 1979; Incharoensakdi *et al.*, 1986; Sakamoto & Murata, 2002). When plants were exposed to excessive levels of salts, glycinebetaine maintained enzyme activity and stabilised the conformation of the enzyme, rubisco (Nomura *et al.*, 1998; Sakamoto & Murata, 2002).

Other solutes, like proline, appeared to play a protective role in plants under drought or salinity stress. In this regard, it has been suggested that proline functions as a source for intracellular osmoregulation, stabilization of proteins, regulation of enzyme activity, regulation of cytoplasmic pH level, and conservation of nitrogen and energy under stress conditions (Stewart & Lee, 1974; McCue & Hanson, 1990; Kalaji & Pietkiwiez, 1993). In addition, Stewart & Lee (1974) proposed that the capacity to accumulate proline under saline conditions is correlated with salt tolerance in some species of halophytes. However, the rise in proline levels with increasing salinity has been associated with a stress reaction rather than any kind of adaptive response (Datta & Ghose, 2003). Furthermore, Datta & Ghose (2003) detected a low concentration of proline in the salt-tolerant *A. marina*.

However, in other studies on similar species demonstrated that proline accumulation in eight salt marsh halophytes increased after very high threshold salinities were reached (Cavalieri & Huang, 1979). The C₄ grasses, *Spartina alterniflora*, *Spartina patens*, and *Distichlis spicata*, for example, accumulated significant levels of proline at salinities of 0.5 M NaCl. The succulents, *Salicornia bigelovii*, *Salicornia virginica* and *Borrchia frutescens* did not accumulate proline until very high salinities (0.7M) were reached (Cavalieri & Huang, 1979). In a field study of *Triglochin maritima* growing under hypersaline conditions, up to 45 % of the total nitrogen of the plant was present as proline (Jefferies & Rudmik, 1991). The leaves of the plant were stunted. Under hypersaline conditions, plants such as *T. maritima* were able to accumulate very high concentrations of proline thereby reducing the size of the plant and the number of leaves. Similar findings were reported for coastal halophytes *Triglochin bulbosa* and *Triglochin striata* (Naidoo, 1994).

Salt toxicity

Salt toxicity in plants is assumed to be associated with this accumulation of Na⁺ ions in leaves after long-term exposure (Mühling & Läuchli, 2002). Salt injury was due to the accumulation of excessive Na⁺ or Cl⁻ (or both) in transpiring leaves, which have exceeded the ability of cells to compartmentalize these ions in the vacuole. Enzyme activity was inhibited when ions accumulated rapidly in the cytoplasm or increased in the cell walls, which consequently caused dehydration in the cell (Flowers & Yeo, 1986; Munns & Passioura, 1984). The build up of Na⁺ or Cl⁻ in the cytoplasm, therefore, is shown to be calamitous if ions in the cell wall did not increase when the vacuole becomes ‘full’ (Munns, 1993). “The rate at which ion concentration has been demonstrated to increase in the cytoplasm which would be much greater than the rate of increase in the vacuole, because the volume of the cytoplasm was much smaller than that of the vacuole” (Munns, 2002).

The effects of salinity on leaf ultrastructure

From an ultrastructural perspective, salinity has been known to affect halophytes (Mitsuya *et al.* 2002). The swelling of organelles such as chloroplasts, Golgi bodies or dictyosomes, mitochondria, and nuclei was commonly associated with plants growing in high salinities or in response to changes in the internal environment of plant tissues (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1975; Kelly *et al.*, 1982; Werker *et al.*, 1983).

Electron microscopic studies revealed that salt-treatment in rice seedlings caused plasmolysis, vesiculation of cellular membranes and degradation of the cytoplasm at the leaf apex (Mitsuya *et al.*, 2002). Mitsuya *et al.* (2000) reported that ultrastructural changes in sweet potato leaf cells (*Ipomoea batatas*) induced by salt stress under light conditions resulted in:

- (a) vacuolation, development and partial swelling of endoplasmic reticulum,
- (b) decrease in mitochondrial cristae and swelling of mitochondria, with an increase in the number of the vesicles released from the Golgi stacks,
- (c) vesiculation and disintegration of tonoplast, and
- (d) degeneration of cytoplasm by the mixture of cytoplasmic and vacuolar matrices.

In the mesophyll tissue, thylakoid membranes of chloroplasts appeared swollen and disintegrated following severe salt stress (Mitsuya *et al.*, 2000). According to Mitsuya *et al.* (2002), degradation of cytoplasm and cell organelles, except thylakoid membranes of chloroplast, arose

as a result of light-independent salt stress, and degradation of thylakoid membranes of chloroplasts in the mesophyll was due to salt-induced oxidative stress.

Yamane *et al.* (2003) observed ultrastructural damage in the leaf chloroplasts of salt-treated rice seedlings induced by NaCl and polyethylene glycol (PEG). They suggested that in salt-treated rice plants, the ionic effects of NaCl induced swelling of thylakoids and the osmotic effects of PEG caused the destruction of the chloroplast envelope. High concentrations of salt also induce changes in mitochondria. Kelly *et al.* (1982) found that in salt-treated plants of *Atriplex halimus* leaves, mitochondria showed no cristal structure, probably due to osmotic disruption and swelling.

Mitochondria and chloroplasts are the main intracellular generators of activated oxygen and damage to these organelles is induced by oxidative reactions (Hernández *et al.*, 1995). Hernández *et al.* (1995) reported that in chloroplasts of pea plants, salt induces oxidative stress and plastoglobuli increase in number and size. The morphology and fine structure of leaves of the seagrass *Zostera capensis* was found to change with high salinity (60 ‰) showing signs of damage to chloroplasts, vacuole size, and plasma membrane (Iyer & Barnabas, 1993).

Extensive lipid accumulation is another discernible effect in some of the chloroplasts of salt-treated plants (Mozafar & Goodin, 1970; Kelly *et al.*, 1982). Thomson (1975) observed that lipid bodies ('plastoglobuli') occur in many types of chloroplasts in a wide range of plants. He suggested that their occurrence was not due to a stress response but to the 'aging' of chloroplasts. In studies reported by Nir *et al.* (1970) water-stressed root-cells showed the accumulation of osmiophilic droplets in the cytoplasm. Similar findings were reported by Poljakoff-Mayber (1975) in which lipid deposits (plastoglobuli) were related to stressed chloroplasts and disruption of membrane components.

Matoh *et al.* (1987) suggested that vacuolation in cells is an adaptive response to compartmentalise Na⁺ ions away from the cytoplasm. Hypersaline conditions induced degradation of cytoplasm due to the mixture of cytoplasmic and vacuolar matrices. Paris *et al.* (1996) observed the presence of active proteases in lytic vacuoles; and maturation of lytic vacuoles featured commonly in senescing leaves of rice plants grown under saline conditions (Inada *et al.*, 1998). An increase in the number of vesicles released from Golgi bodies suggested that Golgi vesicle-proteins accumulated in the vacuole, and therefore salt stress induced the formation of lytic vacuoles as well as the degradation of cytoplasm (Mitsuya *et al.*, 2002).

CHAPTER 3

MATERIALS AND METHODS

3.1. Study site

In Richards Bay Harbour (28° 48'S, 32° 05'E), 172 km north of Durban on the KwaZulu-Natal coast (Fig. 3), an almost pure stand of tall, healthy *A. marina* (6-10 m) dominates the fringe mangroves along the watercourse. Tree height decreases from 6-10 m in the fringe zone to 1-1.2 m in the dwarf zone within an elevational distance of 120 m from the water's edge. Tall, luxurious *A. marina* trees in the fringe site are inundated twice daily by tides with seawater salinities of about 35 ‰ (Fig. 4). The dwarf site supports stunted *A. marina* bordering the barren zone (Fig. 5) and is only inundated by high spring tides with salinities of about 60 ‰ (Naidoo & Chirkoot, 2004). Where *A. marina* meets the barren zone (i.e. lacking any vegetation), the trees are stunted or dwarfed (Naidoo & Chirkoot, 2004). Under the fringe canopy, a thick mat of pneumatophores overlies the rich muddy soil surface. In the dwarf site, however, *A. marina* grows in soils that are salt-encrusted and hardened as a result of infrequent tidal coverage. Pneumatophores in the dwarf site are shorter and fewer in number (Fig. 5) than those in the fringe site (Fig. 4). This investigation forms part of a broader study on factors influencing dwarfing in *A. marina*.

3.2. Collection and selection of plant material

Two sites were selected for sample collection and measurements, one in the fringe zone and the other in the dwarf zone. Leaf material was collected from these two sites. Branches containing fully expanded leaves (second or third from the terminal buds) were selected from five individual trees from each site for measurements. The leaves were quenched in liquid nitrogen on site and transported to the laboratory within 2 hours. Leaves were then freeze-dried for 5 days at -60°C at a vacuum pressure of 10^{-2} Torr in an Edwards Modulyo freeze-dryer. Leaf samples were then milled through a 1mm screen and stored in plastic vials for proline and glycinebetaine analyses.



Fig. 4. Study area showing tall, luxuriant *A. marina* (6-10 m) growing in the fringe site.



Fig. 5. Study area showing stunted *A. marina* (1-1.2 m) growing in the dwarf site.

3.3. Preparation of material for microscopy

3.3.1. Transmission electron microscopy (TEM)

Branches containing fully expanded mature leaves were selected from five individual trees from the two sites, placed in plastic bags moistened with distilled water and kept moist on ice. Plant material was transported within two hours to the laboratory for preparation. Samples were taken from the mid-lamina region of fully expanded, mature leaves. Small segments of leaf material (about 1 mm²) were fixed in 3 % glutaraldehyde buffered with 0.05 M sodium cacodylate buffer (pH 7.2). In addition, 1 % caffeine was added to the fixative to localise secondary metabolites. The material was fixed for 8 hours, and then washed twice for 10 min each in 0.05 M sodium cacodylate buffer. Thereafter, samples were post-fixed in 1 % aqueous osmium tetroxide (OsO₄) for approximately 3 hours at room temperature in a dark cupboard, washed twice for 10 min each in distilled water. The material was dehydrated through a graded ethanol series (20 min each in 50 %, 70 % and 80 % ethanol; 30 min in 95 % ethanol and 2 changes of 30 min each in 100 % ethanol). Material was cleared in two changes of propylene oxide for 20 min each and taken through increasing concentrations of Spurr's (1969) low viscosity resin, diluted with propylene oxide. Samples were then embedded in 100 % resin at 60 °C for 2 days. Resin blocks were trimmed, polished and monitor sections of leaf material ranging in thickness from 0.5 – 2 µm were cut with glass knives and fixed by heat onto pre-cleaned slides with distilled water. The monitor sections were stained with 0.5 % toluidine blue – O made up in 0.1 % sodium carbonate at pH 11.1 (Feder & O' Brien, 1968). After staining for about 20s over heat, sections were washed briefly with distilled water, air-dried and viewed with a light microscope. Cuticle thickness measurements were determined from these sections. Ultra thin (90 nm) sections were cut with a Diatome diamond knife using a Reichert OMU₂ ultramicrotome, collected on uncoated 200 – mesh copper grids, post-stained with 2 % uranyl acetate for 10min, followed by Reynolds's (1963) lead citrate for 8 min. Sections were viewed and photographed with a Jeol 1010 TEM, at 60 kV.

3.4. Scanning electron microscopy (SEM)

Two methods of preparation were employed for SEM, *viz.* chemical fixation and freeze-drying procedures. Leaf segments (5 mm²) were cut from the central portion of fully expanded mature leaf blades.

3.4.1. Chemical fixation

Leaf samples were fixed in 3 % glutaraldehyde buffered with 0.05 M sodium cacodylate buffer (pH 7.2) overnight. The samples were washed with 0.05 M cacodylate buffer for 10 min. Leaf samples were then dehydrated through a graded series of ethanol, and critical point dried with liquid carbon dioxide in a pressurized chamber. Leaves were mounted onto respective brass stubs, with double-sided carbon conductive tape coated with gold for 4min at 30 – 40 milli Amps, in an atmosphere of argon in a Polaron (E 5000) Sputter Coating unit.

3.4.2. Freeze-drying

Leaf segments were placed on a cooled specimen holder and rapidly quenched by plunging in liquid nitrogen, then freeze-dried for 5 days at -60°C at a vacuum pressure of 10^{-2} Torr in an Edwards Modulyo freeze-dryer. Leaves were mounted onto brass stubs with carbon conductive tape, sputter coated with gold and viewed. On the abaxial leaf surface, a thick mat of trichomes obscures the glands and stomata. This mat of trichomes was removed by applying adhesive tape to the abaxial surface for a few seconds and then removing the tape gently.

3.4.3. Freeze-fracture

Pieces of leaf wrapped in parafilm were rapidly quenched in liquid nitrogen, and fractured with the blunt end of a pair of forceps. The fractured samples were then freeze-dried for approximately 5 days at -60°C at a vacuum pressure of 10^{-2} Torr in an Edwards Modulyo freeze-dryer. To view the transverse section of leaves, fractured surfaces of leaves were mounted vertically onto the brass stubs and sputter coated with gold. Both chemically fixed and freeze-dried samples were viewed in a Philips SEM 500 operating at a working distance of 15 mm and an accelerating voltage of 12 kV.

3.5. Salt gland frequency

Five trees were selected from the fringe and dwarf sites and mature leaves (second or third from the terminal buds) harvested. Twenty mature leaves were randomly selected from each site for analyses. The number of salt glands per mm^2 was counted on freeze-dried leaf segments at X 80 magnification selected from three areas (*i.e.* leaf apex, mid-lamina and leaf base) and viewed in a

Philips SEM 500. On the adaxial leaf surface, salt gland frequency was determined on three replicate measurements per leaf area of each of the 20 leaves totaling leaves.

3.6. Proline

For proline determination, leaves were allotted to batches of four comprising 100 leaves per batch. See 3.2. for collection and selection of leaves. Four replicates were done per batch per site.

3.6.1. Extraction of proline from plant material

Proline was then extracted from 0.05 g freeze-dried, milled leaf samples, which was homogenized in 3 ml of 3 % sulphosalicylic acid and the residue removed by centrifugation for 15 min at 12000 rpm. Proline concentrations were determined spectrophotometrically according to the method of Bates *et al.* (1973). One milliliter of the sample extract was reacted with 1 ml acid ninhydrin and 1 ml glacial acetic acid in long screw-cap test tubes. The reaction was terminated by transferring the test tubes to an ice bath. The reaction mixture was extracted with 4 ml toluene and vortexed for 15 s. The chromophore-containing toluene was aspirated from the aqueous phase with Pasteur pipettes and allowed to equilibrate to room temperature. Absorbance readings were recorded at 520 nm using toluene as a blank in a double beam Beckman DU-600 spectrophotometer. Proline was expressed as $\mu\text{g g}^{-1}$ dry mass leaf material.

3.7. Glycinebetine

Leaf samples were allotted to four batches of 50 leaves each. Two replicate measurements were made per site. See 3.2. for collection and selection of leaves.

3.7.1. Extraction of betaines from plant material

Betaines from *A. marina* leaves were extracted according to Naidu (1998) with some modifications as outlined below. Freeze-dried leaf tissue (0.2 g) was placed in 10 ml plastic disposable centrifuge tubes and 5 ml of ice-cold methanol: chloroform: deionised water (MCW: 60: 25: 15) were added. Plant extracts were completely homogenized at 20 000 rpm for 2 to 3 min in ice-cold centrifuge tubes. Cold MCW was added and the centrifuge tube was maintained on ice to reduce heat generation and possible breakdown of solutes. The grinding head was washed with 5 ml deionised water and this was added to the MCW-plant homogenate in the

centrifuge tube. The addition of water served to break the MCW emulsion. The contents of the tube were shaken on a test tube mixer for 10 s and the resulting homogenate was centrifuged at 6000 rpm for 10 min at room temperature. The upper methanol-water (MW) phase was removed, its volume measured and stored below 4 °C in a freezer for the estimation of osmoprotectants.

3.7.2. Preparation of ion exchange resins

The ion-exchange resins were prepared according to Gorham (1984). A strong anion-exchange resin (Amberjet 4400, OH⁻ strongly basic anion exchanger OH⁻ form, quaternary ammonium type), was washed and regenerated in the OH⁻ form on a sintered glass funnel. After washing, surplus water was removed by suction. Similarly, a weak cation-exchange resin (Dowex 50 W-X2 H⁺ 100-200 mesh) was converted to the H⁺ form.

3.7.3. Rapid purification of solutes using ion exchange resins

In some plant species, amino acids or inorganic salts interfere with the measurement of osmoprotectants by co-eluting or eluting close to the peak of interest (Naidu, 1998). To overcome this interference, 1 ml of resin mixture in the ratio of 1:2 cation to anion exchanger, and in a 2- to 3-fold excess of that required to remove inorganic ions and amino acids was placed in a 5 ml plastic syringe and about 1 ml of the plant extract (MW phase) was drawn into the syringe. After shaking for about 5 min, the ion-exchange beads were removed by passage through a small 0.22 µm Millipore filter. The resulting deionised extract or filtrate, which was collected into an auto sampler, was used directly for HPLC (Gorham, 1984).

3.7.4. Betaine standard

Aqueous solutions of anhydrous betaine were prepared in concentrations ranging from 0.01 to 0.2 mg ml⁻¹. Aliquots were applied to the resin mixture in duplicate.

3.7.5 Instrumentation

Two HPLC systems were used, the first consisting of an isocratic HPLC pump, a Rheodyne 7120 injection valve with a 20 µl loop, and a variable-wavelength UV detector. The other comprised a solvent delivery system fitted with a 10 µl loop, a variable UV detector and a computing integrator: Varian Star #1 A-A Workstation (Star Chromatography Workstation version 5.50).

Separations were performed on a 250 mm X 4.5 mm Whatman stainless steel column packed with a strong cation exchange resin Partisil 10-SCX. The buffer consisted of 50 mM potassium dihydrogen phosphate (KH_2PO_4) plus 5% methanol at pH 4.6 which was filtered through 0.22 μm Millipore Durapore filters. There were two replicates per site.

3.8. Quantification of Chlorophyll

Mature leaves were allotted into batches of five comprising 25 leaves per batch. See 3.2. for collection of leaves. Five grams of fresh leaves from each of the five batches per site were weighed and homogenized with a mortar and pestle using 10 ml of 80% acetone. The homogenate was filtered through a paper funnel and then centrifuged for 5 min at approximately 3000 rpm. In a microcentrifuge tube, 0.2 ml of deionised water and 0.8 ml of the crude plant extract were mixed and kept on ice. Total chlorophyll and chlorophylls a and b were determined in a double beam Beckman DU-600 spectrophotometer at wavelengths of 665 and 649 nm, respectively. The blank was 80 % acetone. Concentrations of chlorophyll in 80 % acetone were determined by equations derived by Vernon (1960). There were five replicates per site.

3.9. % Relative leaf water content (RWC)

For RWC determination, mature leaves allotted to batches of nine comprising 25 leaves per batch. See 3.2. for collection and selection of leaves. Leaf RWC was measured by determining fresh mass (FM) and then placing the leaves in beakers of distilled water for 24 h in a dark cupboard. After determining turgid mass(TM), leaves were oven-dried for 72 h at 70 °C and re-weighed to determine dry mass (DM). Leaf relative water content was calculated as $(\text{FM} - \text{DM}) / (\text{TM} - \text{DM})$ and expressed as a percentage. There were two replicate measurements made for the 9 batches of 25 leaves per site.

3.10. Leaf thickness

Leaf thickness was measured in millimeters using a Vernier caliper. See 3.2. for selection of leaves. Leaf thickness was determined from 170 mature, fully expanded leaves from each of the fringe and dwarf sites.

3.11. Cuticle Thickness

Sections from the mid-laminal regions used for light microscopy were selected for the determination of cuticle thickness (μm). The measurements were determined using the Image Analysis 7.0 programme. From each site a total of 60 measurements on randomly selected mature leaves was made.

3.12. Specific Leaf Area

Leaf area was determined using a leaf area meter (C.D. CI-202 Area Meter). See 3.2. for collection and selection of leaves. There were two replicate measurements made for the 9 batches of 25 leaves per site as described for 3.9. %RWC.

3.13. Ion Analyses

Approximately fifty mature, fully expanded *A. marina* leaves were harvested as described for 3.2. Leaves were rinsed in distilled water to remove surface salt and dried in an oven for 48 hours at 70 °C. Leaf samples were then ground in an agate mill through a 1mm screen and stored in plastic vials. Subsamples were dry-ashed in a muffle furnace at 450°C and dissolved in 1M HCl. Concentrations of Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cu^{2+} , Mn^{2+} and Zn^{2+} were determined by atomic absorption spectroscopy (Varian Spectra AA-10, Mulgrave, Australia), P by the molybdenum blue procedure and N by the automated Dumas dry combustion method, using a LECO CNS 2000, Leco Corporation, Michigan, USA (Matejovic, 1996).

3.14. Statistical Analyses

Data from all measured parameters between fringe and dwarf sites were subjected to unpaired *t*-tests using GraphPad InStat Version 3.00 (Mustek) and GraphPad Prism Version 2.00.

CHAPTER 4

RESULTS

4.1. ANATOMY AND MORPHOLOGY

The lamina of fringe mangrove leaves has a relatively smooth adaxial surface with prominent salt glands interspersed at regular intervals (Fig. 6). A dense indumentum of peltate non-glandular hairs or trichomes is found on the abaxial surface (Figs. 7, 8). Typically, the leaf comprises a relatively thick adaxial cuticle (Fig. 10), regularly shaped epidermal cells (Figs. 11, 24, 26), a multi-layered hypodermis and well-developed mesophyll tissue (Fig. 11). The latter comprises long symmetrical palisade mesophyll approximately 2-4 cell layers and oval-rounded to isodiametric spongy mesophyll cells (Fig. 11). The adaxial epidermal cells were often larger than those of the abaxial cells (Fig. 11). Leaves are hypostomatous (Figs. 9, 11). Abundant salt glands are distributed on the abaxial leaf surface partially obscured by the overlapping uniseriate peltate hairs (Figs. 7, 8, 10). Salt crystals were frequently dispersed on the trichomes (Figs. 7, 8).

In both fringe and dwarf mangrove leaves, scanning electron microscopy (SEM) of the adaxial leaf surface revealed the presence of conspicuous salt glands randomly distributed at regular intervals. The glands were either deeply sunken within crypts as in dwarf mangrove leaves (Figs. 17, 19) and/or generally protruding from crypts as was evident in leaves of fringe mangroves (Fig. 18). In both fringe and dwarf mangrove leaves, salt crystals were abundant on the surface and surrounding glands (Figs. 17, 19 arrows). Secretory material with amorphous structures was also found on and around the salt glands, occluding and embedding the glands within the crypts (Figs. 15-17). A thick, mat of densely clustered non-glandular hairs, which concealed the salt-exuding glands, covered the abaxial surface (Fig. 8). Trichomes had a multicellular sclerotic body, which distally appeared to give rise to an unbranched filamentous body (Tomlinson, 1986) (Fig. 8).

In *A. marina* leaves, the most prominent feature of the adaxial leaf surface was the presence of salt glands “scattered in individual pits” (Fig. 6). SEM revealed the various dimensional views of a typical salt gland. The surface is contoured along the lines of the glands (Figs. 6, 10). Generally, the upper portion of secretory cells of glands has a thin, minutely perforated cuticle enclosing the cells. Quadrants of the secretory cells of the protruding gland were commonly

PLATE 1

Figure 6. SEM of surface of fringe mangrove leaf showing general morphological features. Relatively smooth adaxial leaf surface covered by thick cuticle with sunken salt glands (SG) within crypts occurring at regular intervals. Note ball of secretion over gland (arrow) and salt crystals (short arrows) on surface.

Bar = 10 μm

Figures 7-8. SEM of abaxial leaf surface of fringe mangrove showing trichomes.

Fig. 7. Thick mat or indumentum of multicellular peltate trichomes (t). Note the dense overlapping trichomes with concave heads (h).

Bar = 10 μm

Fig. 8. Freeze-fractured leaf showing abaxial salt gland (SG) occluded by overlapping trichomes (t). Note thin abaxial cuticle on lower epidermis (arrow). The trichomes are comprised of a stalk (St) and a broad concave head (h).

Bar = 10 μm

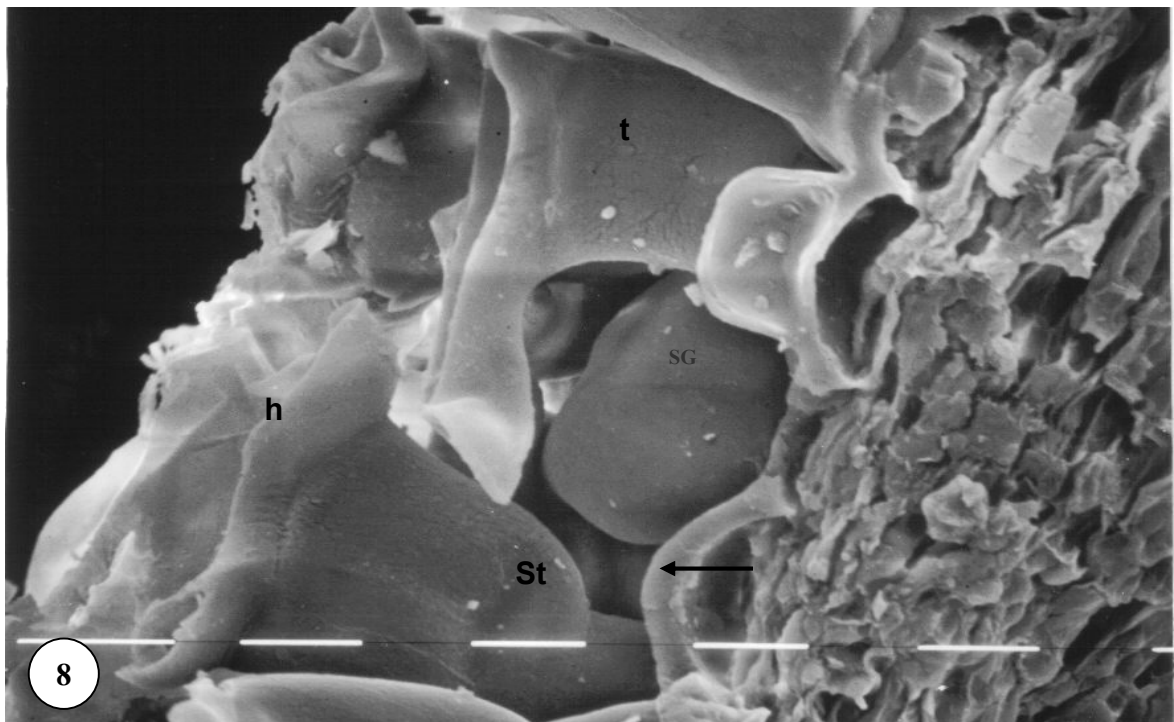
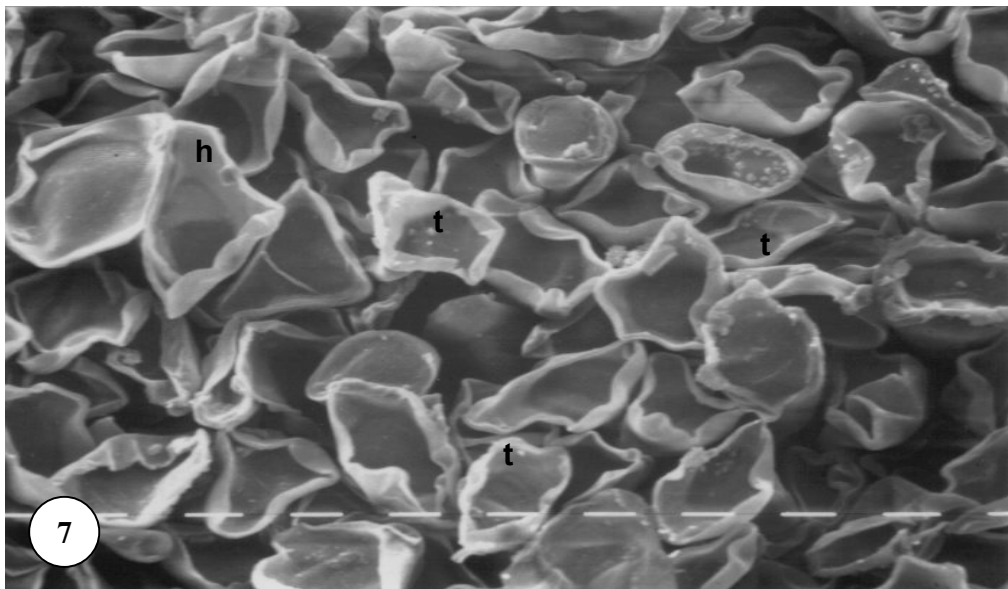
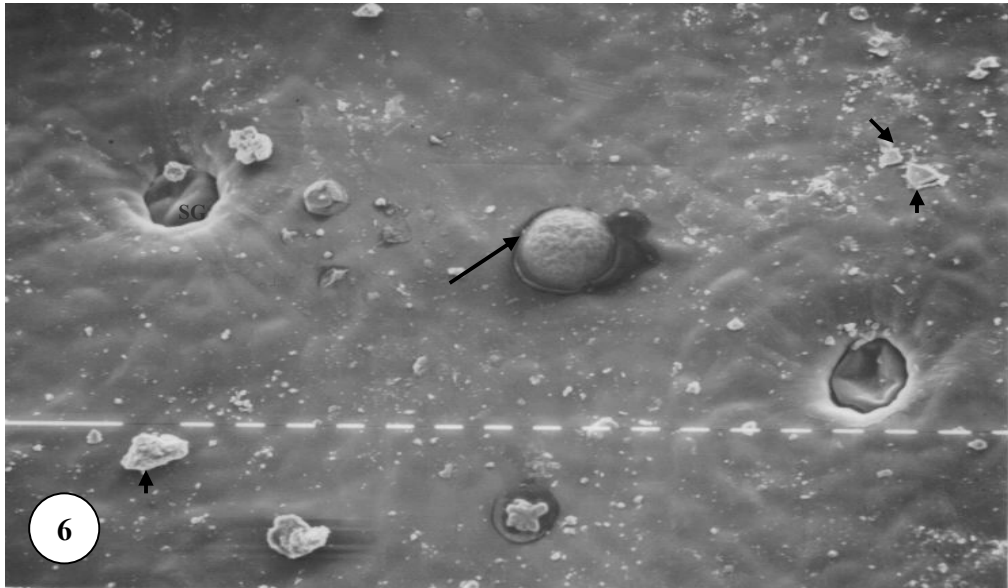


PLATE 2

Figure 9. SEM of abaxial surface of fringe mangrove leaf showing stalks of trichomes removed (arrowhead), salt glands (SG) and open stomata (s) found at regular intervals between trichomes.

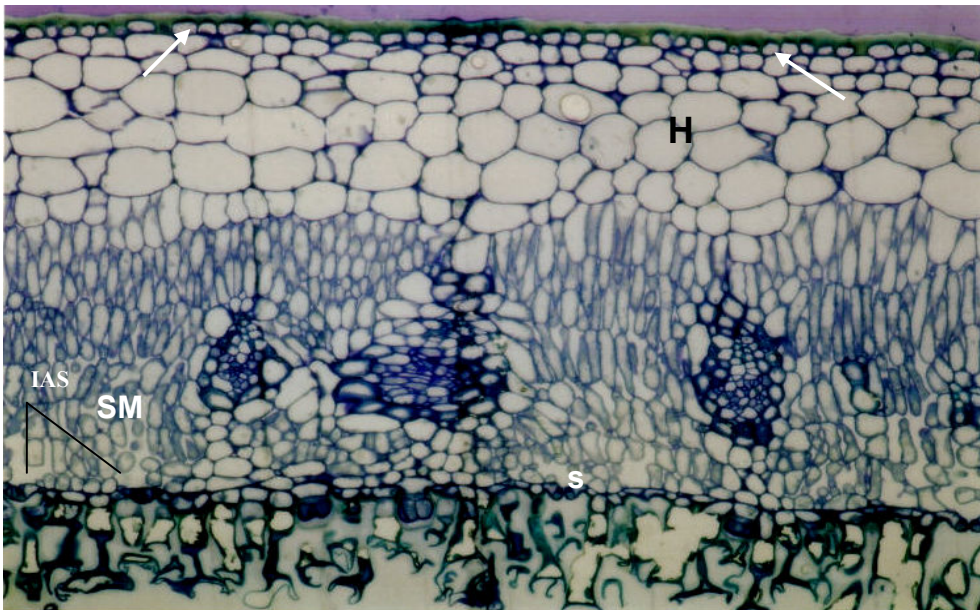
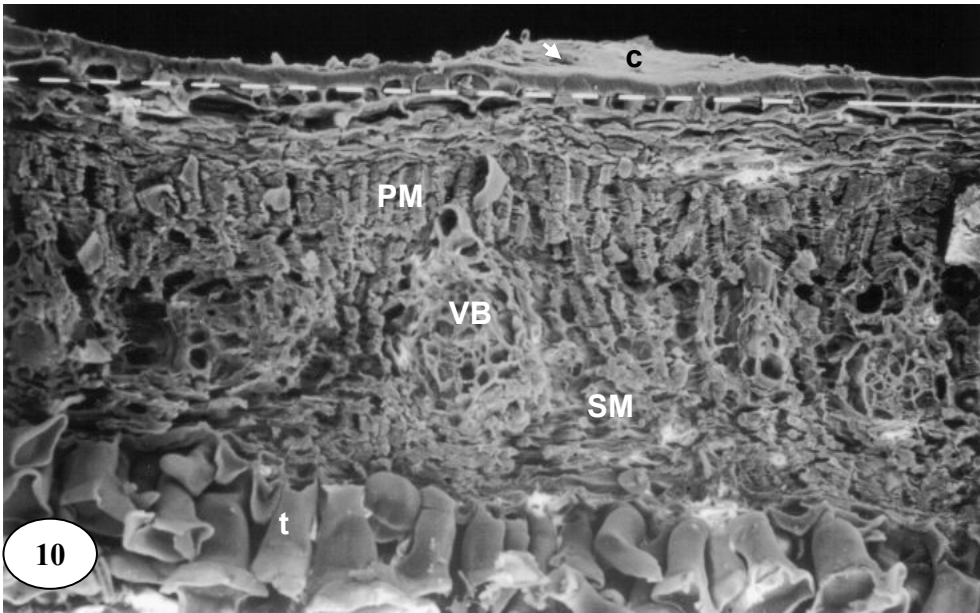
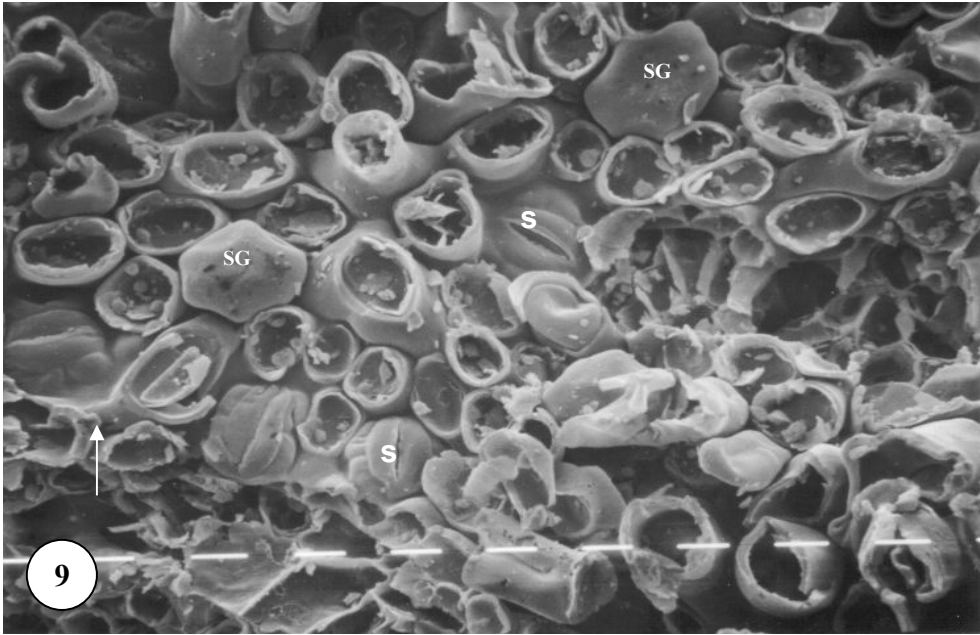
Bar = 10 μm

Fig. 10. SEM of freeze-fractured fringe mangrove leaf showing thick cuticle (c) palisade mesophyll (PM), vascular bundle (VB), spongy mesophyll (SM) and trichomes (t) on the abaxial surface. Note protruding salt gland (arrowhead) on abaxial surface.

Bar = 10 μm

Fig. 11. Light micrograph of toluidine blue stained transverse section of the mid-laminal region of fringe mangrove showing general leaf anatomy and morphology. Note regularly shaped epidermal cells (arrows), tightly appressed hypodermal cells (H) comprising 5-6 layers, with well-developed mesophyll tissue. Intercellular air spaces (IAS) associated with spongy mesophyll (SM). Multicellular abaxial salt glands (SG) and stomata (s) located at regular intervals and flanked by a dense indumentum of peltate trichomes (t).

Bar = 10 μm



visible in fringe mangroves (Figs. 12, 13). In dwarf mangrove leaves, the cuticle covered the surface extensively, occasionally thickened, enveloping and coalescing towards the pit/crypt of the gland (Fig. 15). In both leaf types, large crystalline deposits of salt and salt crusts formed as a result of salt secretion on gland surfaces (Figs. 14, 15) as well as on the side walls of the crypts in the region of the glands (Figs. 13, 14).

Salt glands were abundantly distributed on the abaxial surface of leaves of fringe mangroves (Fig. 9). Generally, the abaxial glands were not sunken but obscured by the dense indumentum of trichomes (Fig. 8). The salt glands were visible when the mat of trichomes was physically removed by using adhesive tape (*see 3.4.2. Freeze-drying procedure*) (Fig. 9). A number of surrounding trichomes obscured the abaxial gland as shown in Fig. 20. Freeze-fracture of a fringe mangrove leaf exposed the outer walls of the secretory cells, revealing its structure (Fig. 21). The gland appeared large and bulb-like with distinct quadrants. Salt crusts commonly formed on the cutinised surface of the gland (Fig. 21).

SEM revealed that although the glands in leaves of fringe mangroves largely occupied individual crypts (Figs. 14, 18, 22), they regularly appeared rounded and protruding as compared to the frequently depressed glands as observed in dwarf mangrove leaves (Figs. 16, 19). Glandular degeneration was also evident in leaves of dwarf mangroves (Fig. 23). The adaxial leaf blades of dwarf mangroves exhibited a heavier deposition of salt crystals and more sunken glands as compared to salt glands of fringe mangrove leaves.

Numerous stomata were found distributed amongst the salt glands and the trichomes of both fringe and dwarf mangrove leaves. Leaves were hypostomatous i.e. stomata confined to the abaxial surfaces only (Fig. 9). The stomatal apparatus consisted of two kidney-shaped guard cells each supported by large, swollen subsidiary cells located laterally to the adjacent guard cells (Figs. 9, 61).

4.1.2. ULTRASTRUCTURAL STUDIES

TEM elucidated several distinct differences in leaf ultrastructure between fringe and dwarf mangrove leaves, with respect to cuticle thickness, chloroplasts and mitochondria of mesophyll tissue and the salt glands. These differences are discussed separately in the following sections.

Cuticle and epidermis

In the epidermal cells of both leaf types, a large vacuole occupied most of the cell volume. (Figs. 26, 27) Within the parietal cytoplasmic layer, various organelles were found (Fig. 26, arrows).

PLATE 3

- Figures 12-14.** SEM of adaxial leaf salt glands of fringe mangroves.
- Fig. 12.** Salt gland comprised of four visible quadrants (arrowheads). Note salt secretions around gland (arrows).
Bar = 10 μm
- Fig. 13.** Salt gland (SG) protruding from crypt (arrow). Salt secretions (arrow) and thick cuticular (c) material around gland.
Bar = 10 μm
- Fig. 14.** Salt gland (SG) in crypt (Cr) covered with a salt crust (arrows). Thick cuticular or secretory material is partially occluding the gland (arrowheads).
Bar = 10 μm
- Figures 15-17.** SEM of adaxial salt glands in dwarf mangrove leaves showing abundance of salt secretions.
- Fig. 15.** Thick layer of cuticular or secretory material and salt exudates (arrow) enveloping gland.
Bar = 10 μm
- Fig. 16.** Amorphous secretory material (arrow) emanating from salt gland (SG) onto surface. Note ball of secretion (bs) over gland.
Bar = 10 μm
- Fig. 17.** Thick globules of secretory substance (b) completely occludes gland (SG) sunken within crypt.
Bar = 10 μm

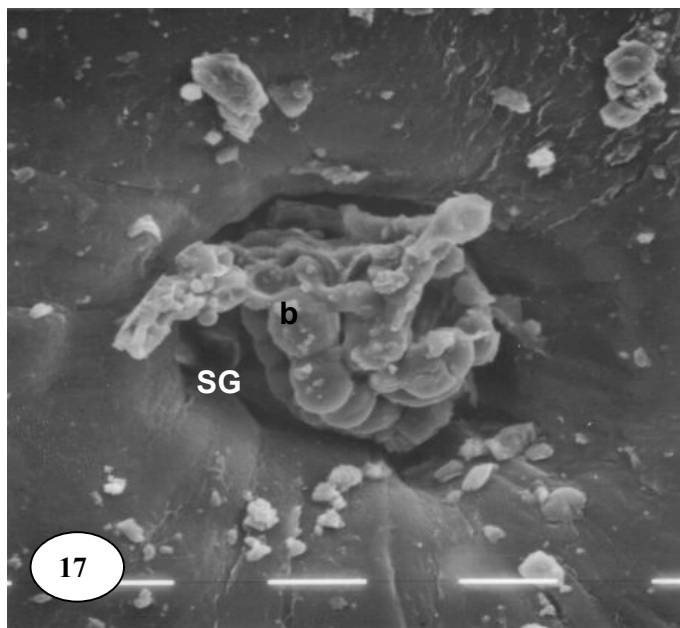
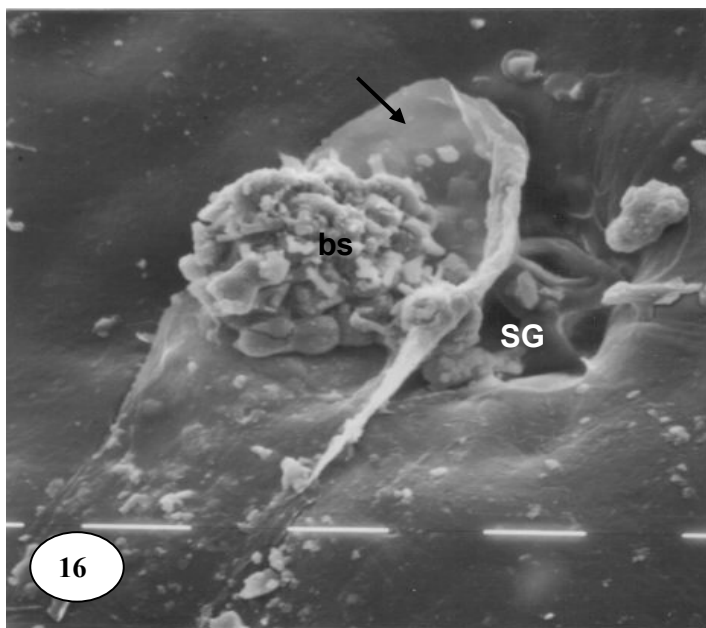
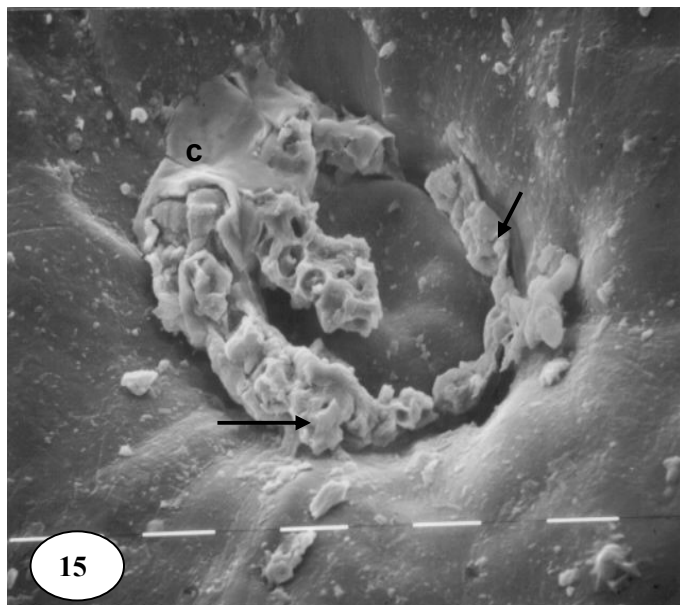
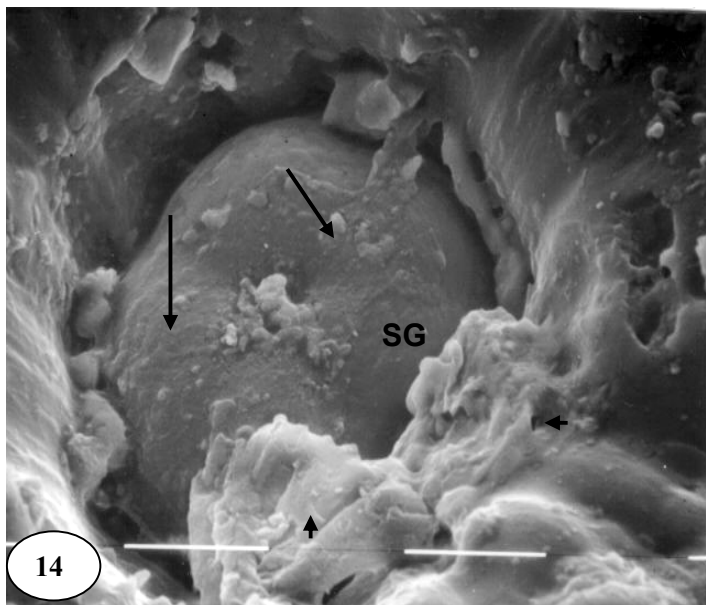
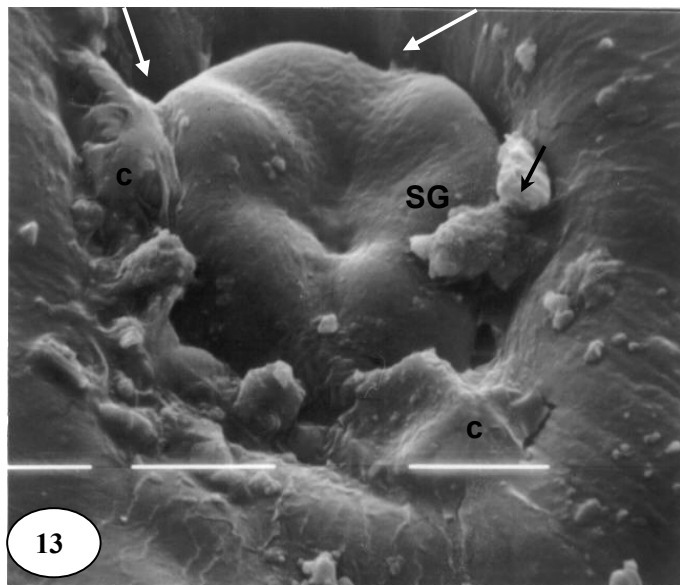
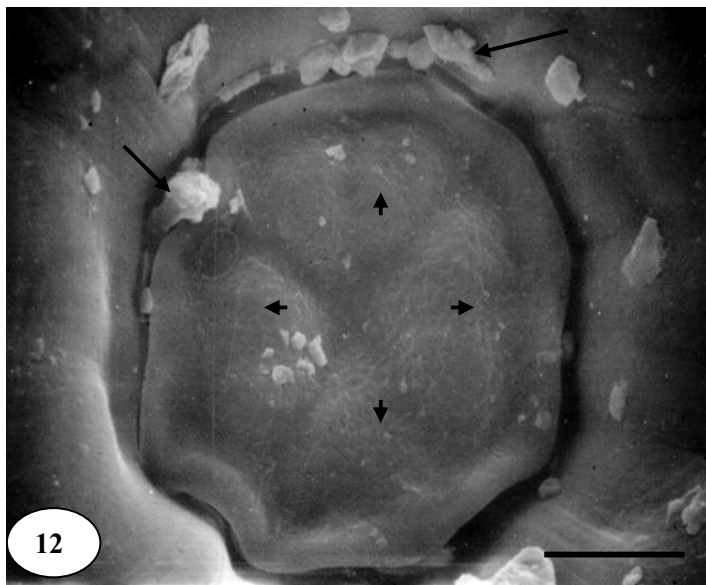


PLATE 4

Figures 18-19. SEM showing differences in adaxial leaf salt glands between fringe and dwarf mangrove leaves.

Fig. 18. Fringe mangrove leaf: Salt gland (SG) in crypt. Note cavity in centre of gland partially occluded by secretory or cuticular material (arrow).

Bar = 10 μ m

Fig. 19. Dwarf mangrove leaf: Salt gland (SG) sunken in crypt (Cr) and heavy deposition of salt crystals (arrows) around gland. Depressions (short arrow) on the gland demarcate the quadrants (arrowhead).

Bar = 10 μ m

Figures 20-21. SEM of abaxial leaf salt glands of fringe mangroves.

Fig. 20. Salt glands (SG) exposed after removal of peltate trichomes.

Bar = 10 μ m

Fig. 21. Freeze-fractured surface showing abaxial salt gland (SG). Note salt crust (arrow) partially covering gland surface.

Bar = 10 μ m

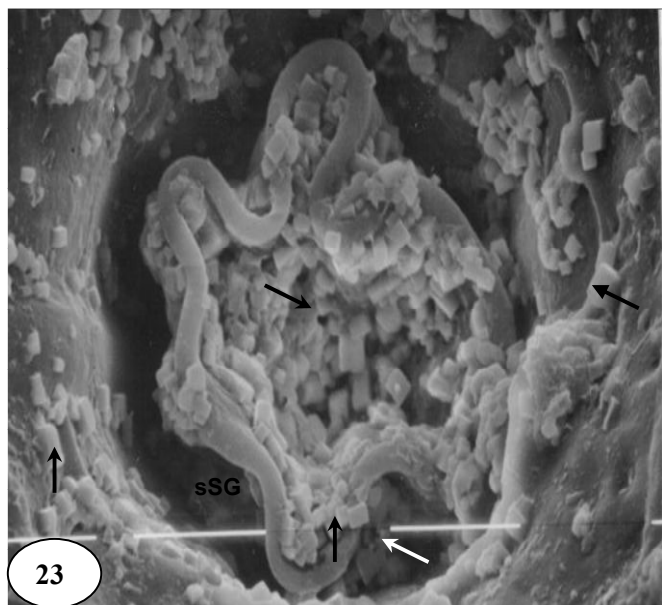
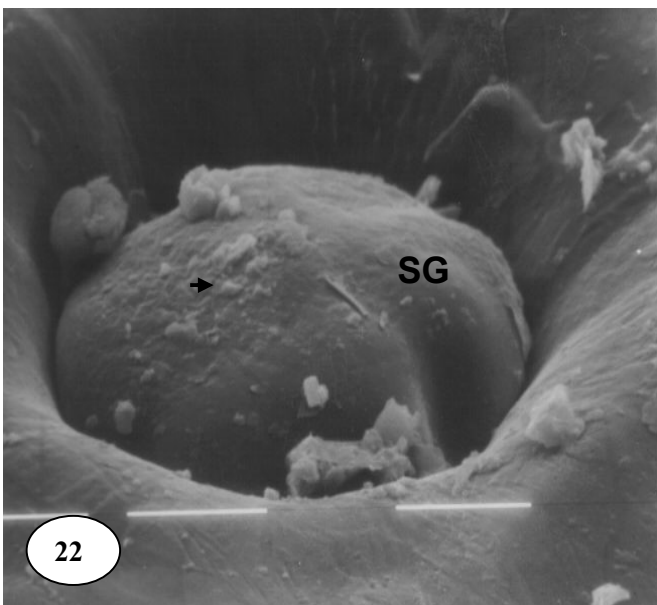
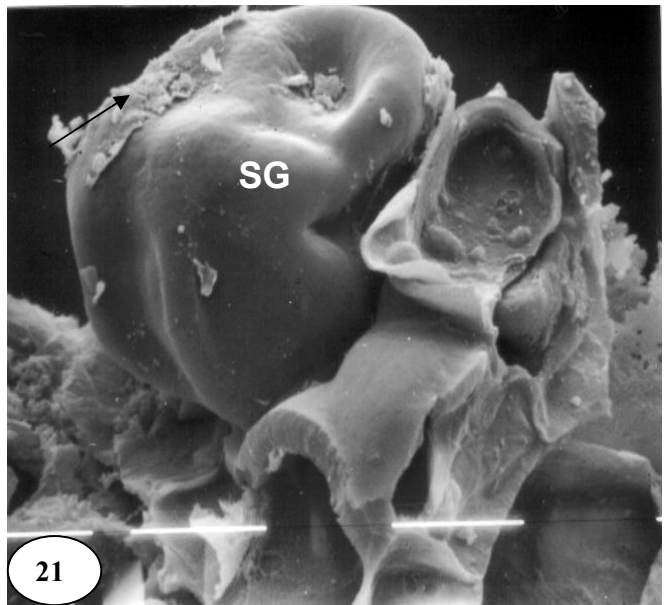
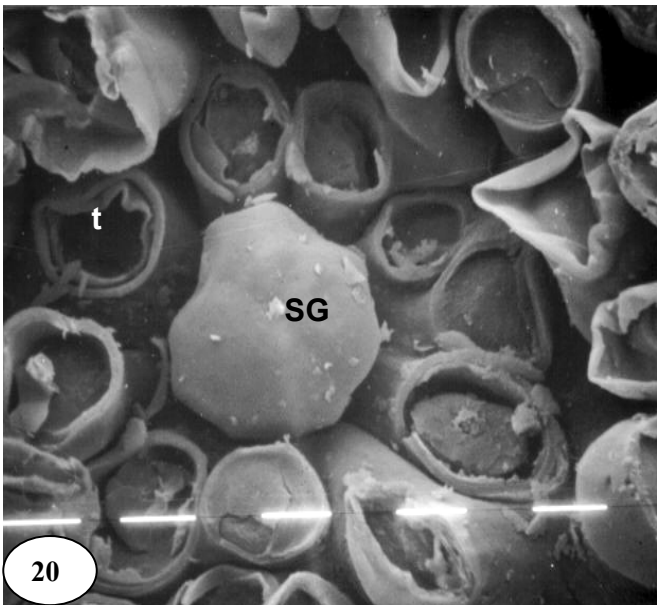
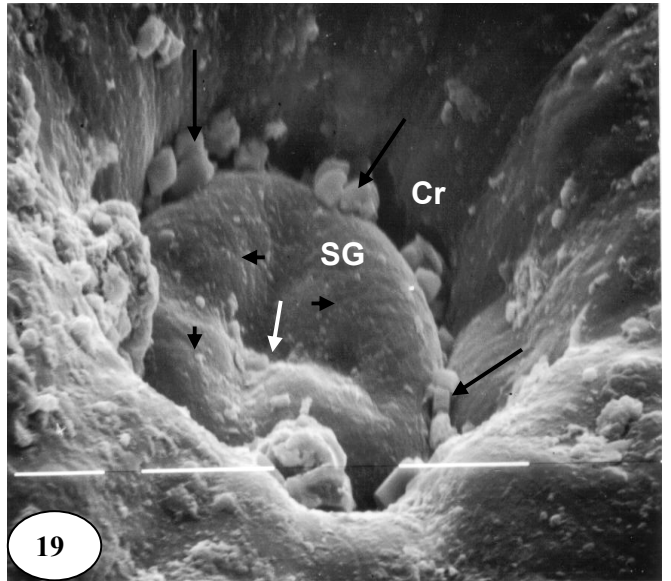
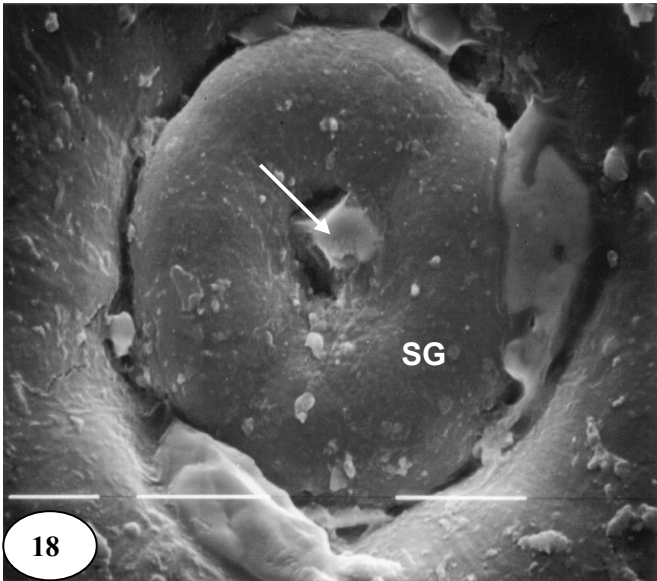
Figures 22-23. SEM of adaxial leaf showing fringe and dwarf mangrove leaf salt glands.

Fig. 22. Fringe mangrove leaf: typical, mature salt gland (SG) protruding from cavity.

Bar = 10 μ m

Fig. 23. Dwarf mangrove leaf: Senescing or degenerating salt gland (sSG) sunken within crypt in a state of collapse. Cuboidal salt crystal deposits (short arrows) encrusted on gland and upper surface.

Bar = 10 μ m



Nuclei were frequently visible in sections of these cells, occupying the peripheral regions of the cells (Fig. 26).

The adaxial cuticle of fringe mangrove leaves was thick (mean value = $12 \mu\text{m} \pm 0.627$) and waxy and the epidermal cells were large, vacuolated and appeared thick-walled (Figs. 24, 26). However, dwarf mangrove leaves exhibited a substantially thicker cuticle (mean value = $19 \mu\text{m} \pm 0.823$) (Fig. 27) and the epidermal cells were predominantly irregular in shape or appeared discontinuous (Fig. 25).

Hypodermis

In both fringe and dwarf mangrove leaves, the hypodermis appeared multi-layered (5-9 layers), comprising closely appressed, vacuolated cells that were much larger than epidermal cells (Figs. 24, 25). Hypodermal cells ranged in shape from oval to cuboidal (Figs. 24, 25, 26). Both dwarf and fringe mangrove leaves exhibited a thick multi-layered hypodermis (Figs. 24, 25). The hypodermal cells are highly vacuolated in both leaf types. The walls of the hypodermal cells in leaves of fringe mangroves appeared thick and well formed (Fig. 28). Chloroplastic organelles were distributed within the cytoplasm along the parietal walls of these cells (Fig. 26, long arrow). However, the inner tangential walls of dwarf mangrove leaves were particularly wavy and thicker in appearance (Figs. 29, 30) than those of the fringe mangrove leaves (Fig. 28).

Mesophyll (palisade and spongy)

Generally, in fringe mangroves, mesophyll tissue was well developed with non-assimilatory layers found below the thick, colourless hypodermal layers. The mesophyll tissue was well differentiated in the dorsiventral leaf and consisted of the adaxial elongated palisade cells arranged parallel to each other (Fig. 31) and oval to round abaxial spongy mesophyll cells (Fig. 37). Inter-cellular air spaces were closely associated with spongy mesophyll cells (Fig. 37). Chloroplasts were prevalent in the palisade and spongy mesophyll cells (Figs. 32, 38). Mesophyll cells were largely vacuolated. The single large vacuole present in mesophyll cells compressed the nucleus and the residual, metabolically active cytoplasm into a thin parietal layer against the cell walls (Figs. 31, 32, 37).

PLATE 5

Figures 24-25. Light micrographs of toluidine blue-stained transverse sections of *A. marina* leaves showing differences in adaxial cuticle thickness of fringe and dwarf mangroves.

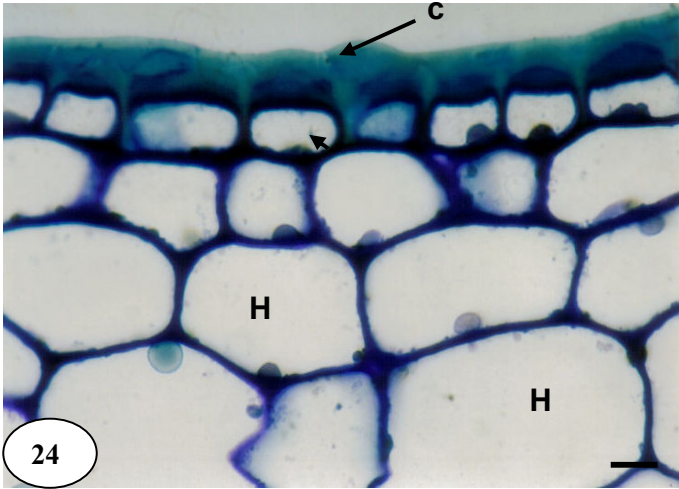
Fig. 24. Fringe mangrove leaf: Note outer layers of leaf tissue showing regularly arranged epidermal cells (arrowheads) below a relatively smooth cuticle (c) (arrow). Hypodermal cells (H) appear colourless and highly vacuolated.
Bar = 10 μ m

Fig. 25. Dwarf mangrove leaf: Note outer layers of leaf showing significantly thicker adaxial cuticle (c). Epidermal cells generally appeared discontinuous and irregular in size and shape (short arrows).
Bar = 10 μ m

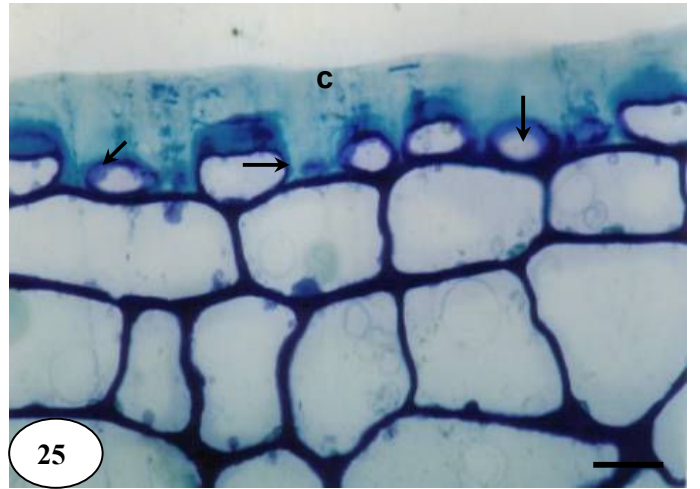
Figs. 26-27. TEM of adaxial surface of fringe and dwarf mangrove leaves.

Fig. 26. Adaxial cuticle (c) and epidermal cells (E) of fringe mangrove leaf. Note thick cuticle overlying large regularly shaped epidermal cells. A thin parietal cytoplasmic layer occupied the peripheral regions of the epidermal cells (arrowheads). The nucleus (N) comprising densely arranged chromatin is situated in the lower region of the cell, bordered by elliptical-shaped chloroplasts (Cl). The epidermal cells had a large vacuolar space (Va). Hypodermal cells (h) occurring below upper epidermal layer.
Bar = 2 μ m

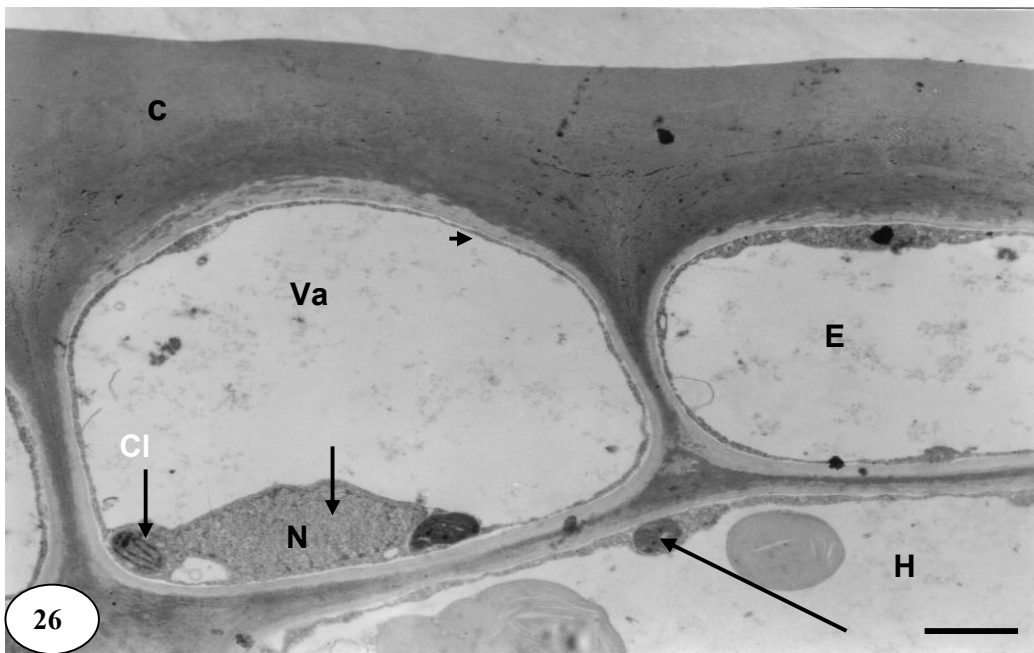
Fig. 27. Dwarf mangrove leaf exhibited a significantly thicker adaxial cuticle (c) than that of fringe mangroves. The epidermal cells were generally irregular in shape and vary in size (arrows).
Bar = 2 μ m



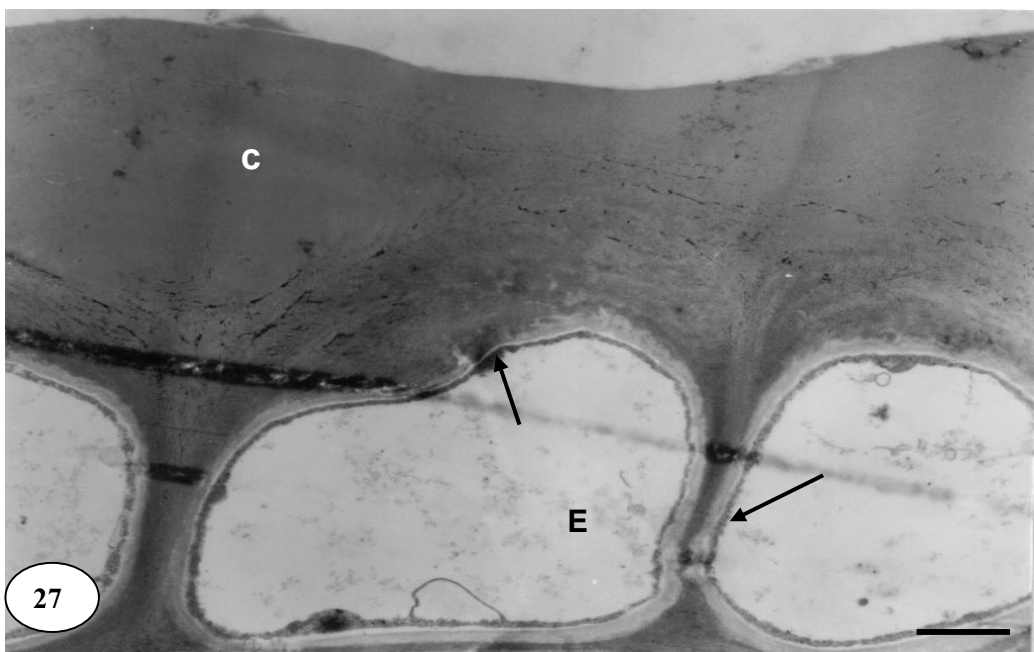
24



25



26



27

PLATE 6

Figures. 28-30. TEM of hypodermal cell walls of fringe and dwarf mangrove leaves.

Fig. 28. Fringe mangrove leaves exhibited thick, well-defined and distinct hypodermal cell walls (CW). Note uniform arrangement of cellulose microfibrils within the cell walls (arrows). Chloroplasts (Cl) of hypodermal cells are commonly distributed along the inner tangential walls.

Bar = 500 nm

Fig. 29. Dwarf mangrove leaf: Note withdrawal of plasma membrane and cytoplasm from the hypodermal cell wall (arrows). In the cytoplasm, vesicles (v) appeared to be formed as a result of fusion with the plasma membrane (plasmalemmasomes) and in many instances seemed to be released into the large vacuolar space (Va) of the hypodermal cells. Plasmodesmata connecting neighbouring cells appeared wavy and disjointed (long arrow). Smaller vesicles (v) are compartmentalised within larger vesicle forming a multivesicular structure (MV), which may be due to the high salt condition.

Bar = 200 nm

Fig. 30. Dwarf mangrove leaf: Note presence of wide extracytoplasmic space (Ec) in the vicinity of the cell walls as a result of complete withdrawal of the plasma membrane (PM) and cytoplasm from the cell wall (CW).

Bar = 200nm

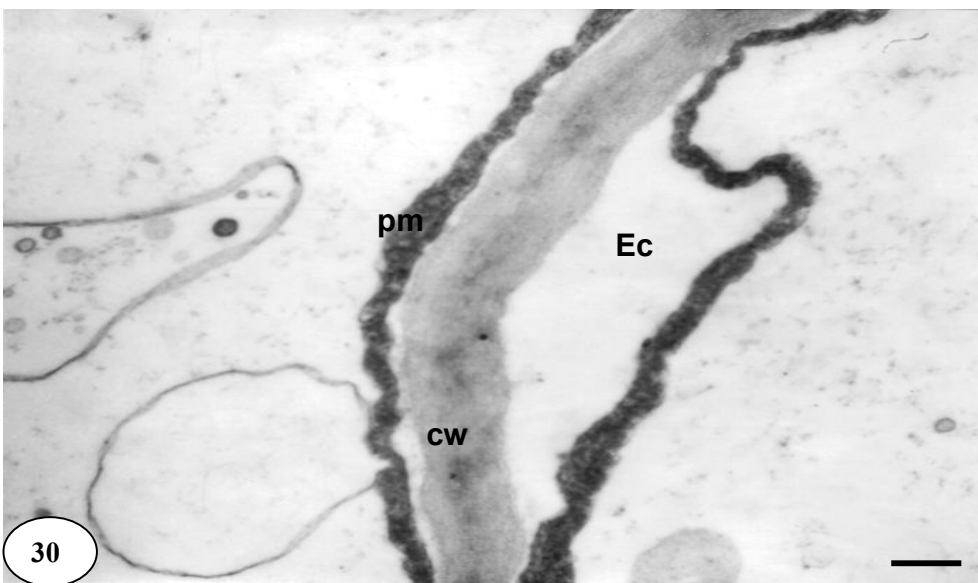
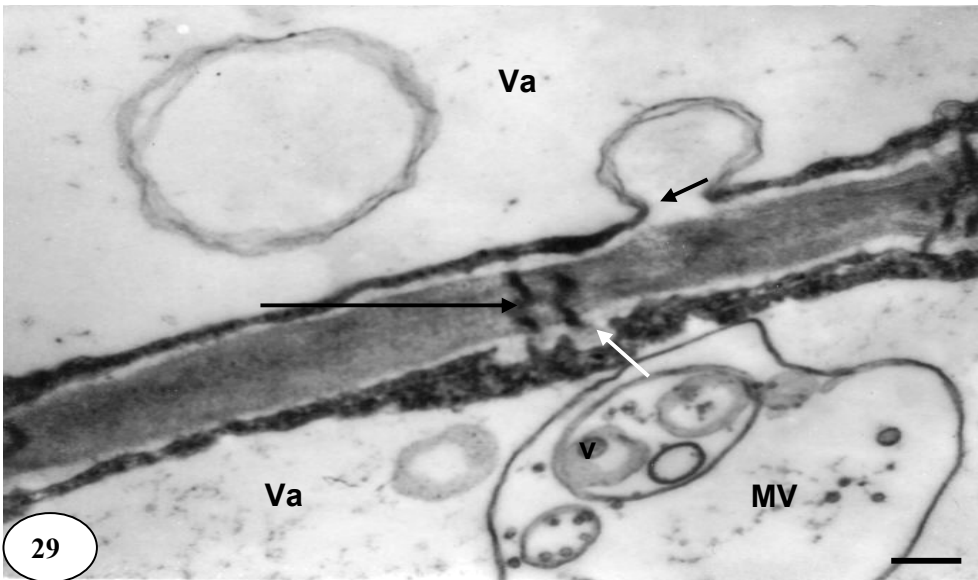
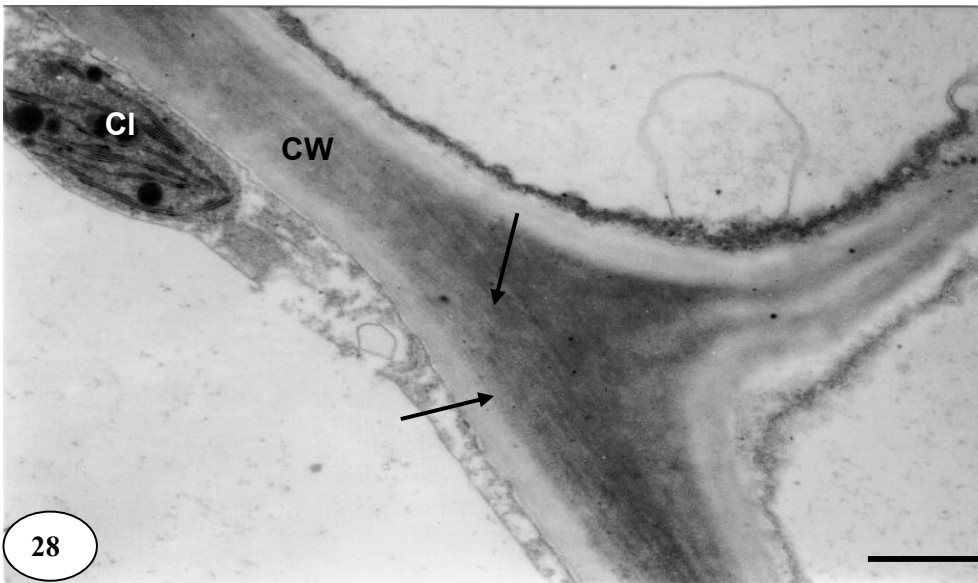


PLATE 7

- Figures 31-32.** TEM of palisade mesophyll cells from fringe mangrove leaves.
- Fig. 31.** Large vacuolar space (Va) with peripherally situated nucleus (N) and well-defined nucleolus. Mitochondria (M) generally located in the vicinity of chloroplasts (Cl) in the cytoplasm.
Bar = 2 μm
- Fig. 32.** Large nucleus (N) with dense chromatin network enclosed by double nuclear envelope. Chloroplasts (Cl) contained very large starch grains (sg) (arrow). Note plasma membrane closely following contours of cell wall (short arrow).
Bar = 1 μm
- Fig. 33.** Ultrastructure of palisade mesophyll cells in dwarf mangrove leaves. Withdrawal of plasma membrane (arrowheads) from cell wall (cw) as well as displacement of chloroplast (Cl) from the plasma membrane boundary (white arrowheads). Multivesicular structures (MV) occupied large vacuolar space (Va). Chloroplasts (Cl) appeared to be swollen with few granal stacks (short white arrows). Note formation of numerous, small plasmalemmasomes (vesicles associated with the plasmalemma) between cell wall and disintegrated plasma membrane in the extracytoplasmic space (thin arrows).
Bar = 2 μm

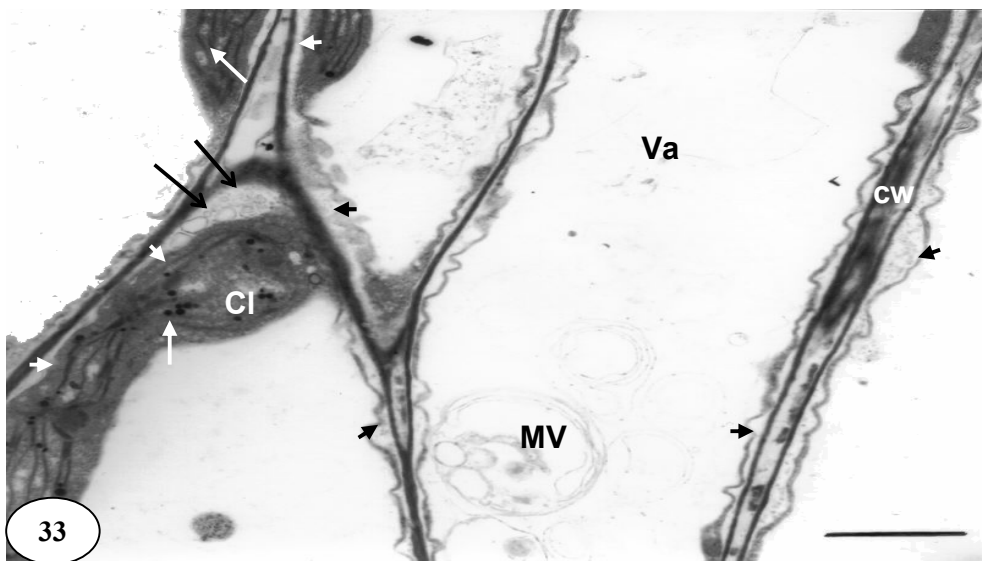
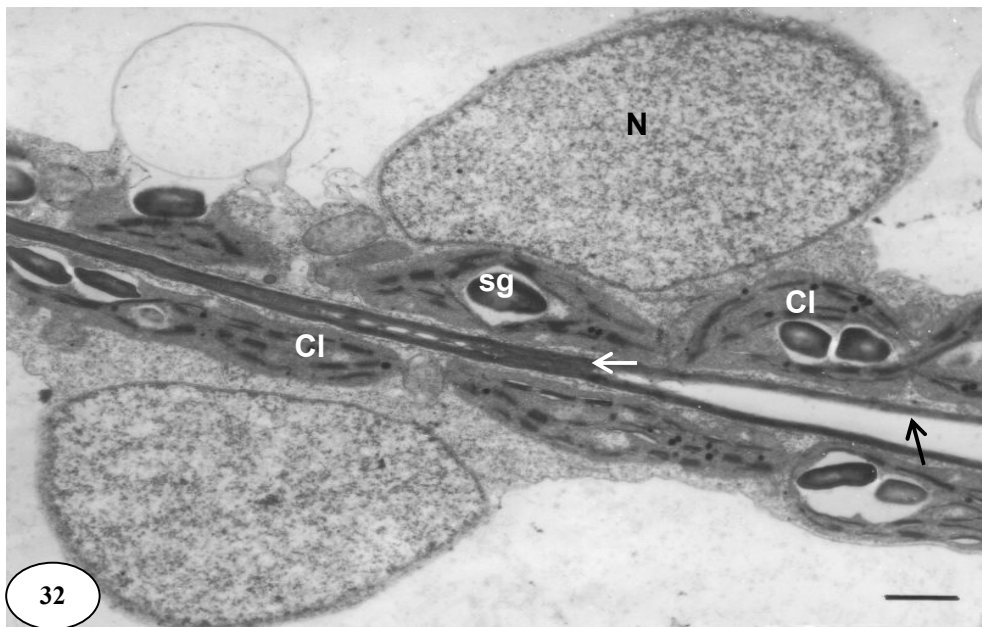
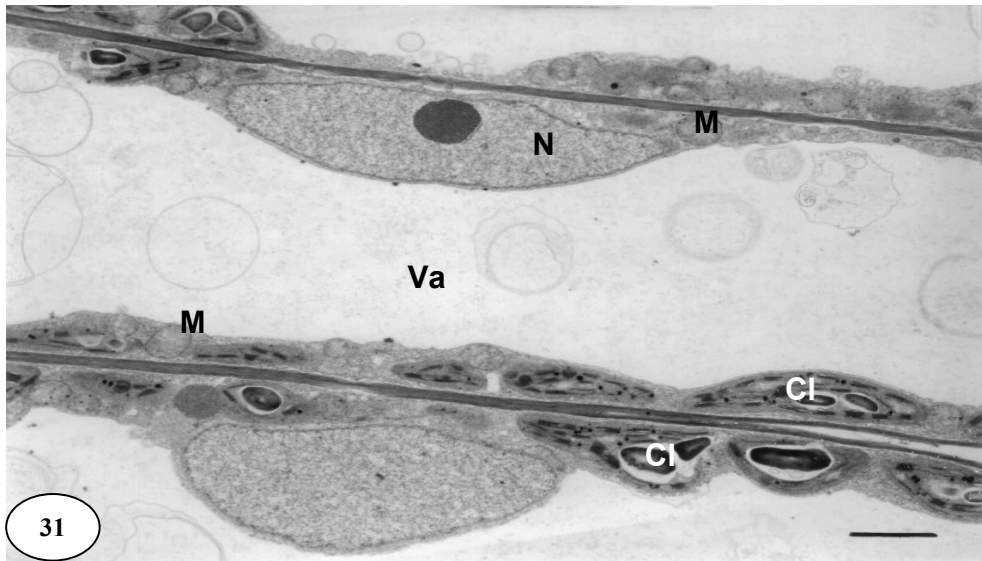


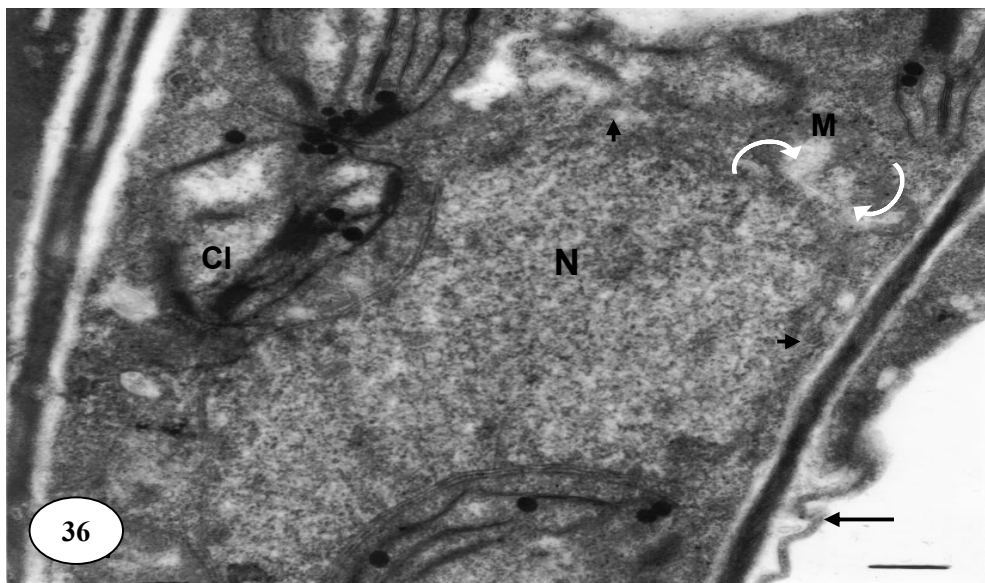
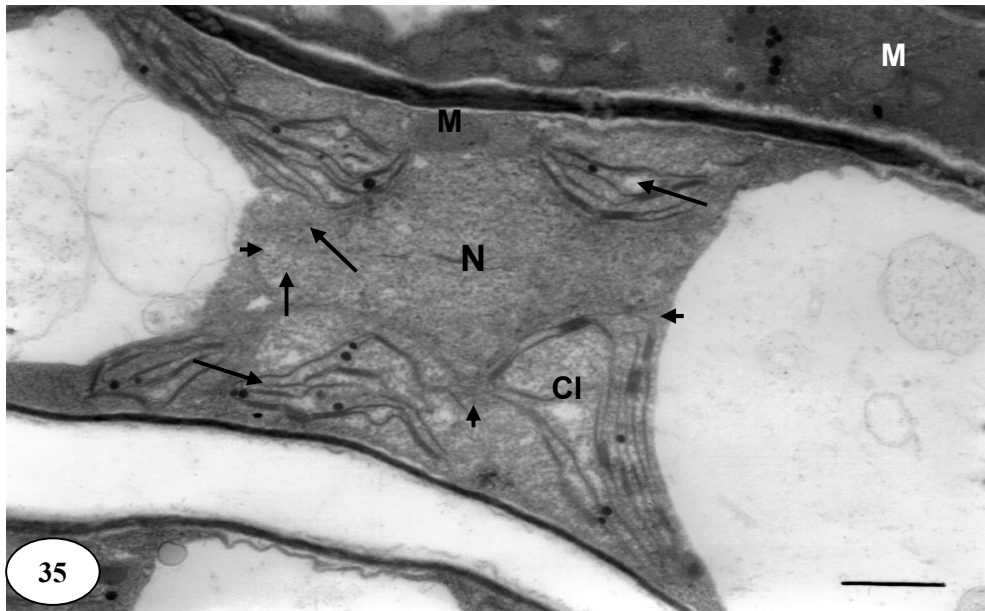
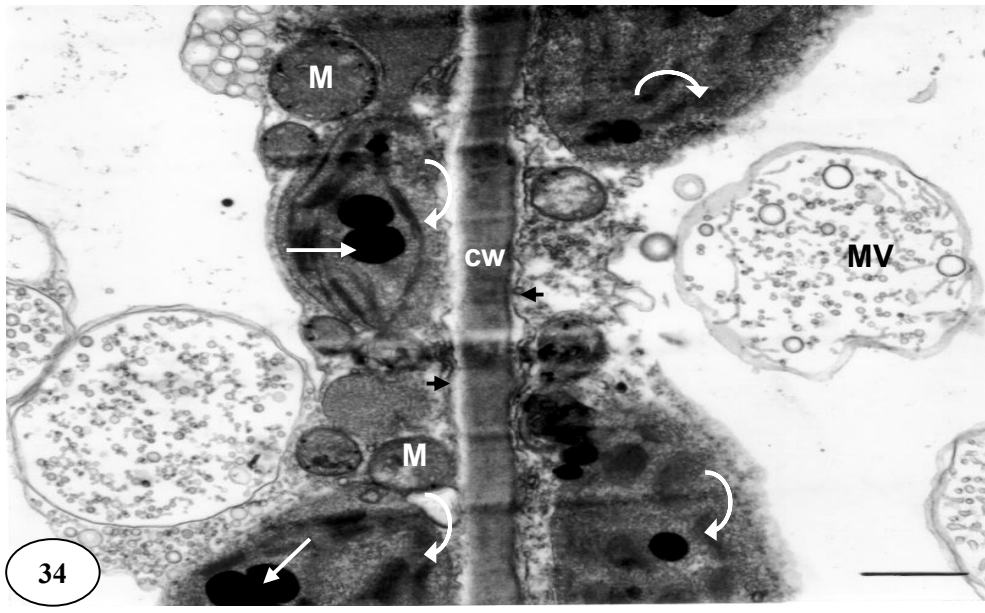
PLATE 8

Fig. 34. Ultrastructural detail of palisade mesophyll cells in dwarf mangrove leaf. Numerous multivesicular structures (MV) accumulate in the vacuole (Va). Plasma membrane (arrowhead) appeared to be distorted and displaced from the cell wall. Mitochondria (M) closely associated with chloroplasts which appeared to be rounded in shape and possessed dilated cristae. Note unusually large plastoglobuli (white arrows) appeared to be fusing with one another. There is a reduction in the size of the granal stacks (curved arrows)
Bar = 500 nm

Figures 35-36. Ultrastructural detail of palisade cells in dwarf mangrove leaf.

Fig. 35. Chloroplasts appeared amoeboid in shape. Note distortion and disintegration of chloroplast membranes (arrows). Indistinct chloroplast envelope (arrowheads), lack of starch grains and centrally located nucleus (N).
Bar = 1 μ m

Fig. 36. Note withdrawal of plasma membrane from wall (arrow) and nuclear envelope of nucleus (N) seemed to be indistinguishable (arrowheads). Chloroplasts (Cl) appeared highly swollen with little membrane integrity. Grana stacks were few; thylakoids appeared indistinguishable from each other. Mitochondria (M) were disorganised and appeared to lack cristae. Note amoeboid deformation of chloroplast.
Bar = 500 nm



Differences in palisade mesophyll cell ultrastructure between fringe and dwarf mangrove leaves.

Palisade cells in leaves of fringe mangroves seemed to be continuously joined to each other; the plasma membranes were closely appressed to the cell walls (Fig. 32). The cytoplasm constituted a thin dense layer rich with ribosomes and closely appressed to the cell wall by the large central vacuole which occupied the bulk of the cell volume (Fig. 31). The parietal cytoplasmic layer was populated with mitochondria, chloroplasts, large nuclei, ribosomes, microbodies, Golgi bodies, ER and vesicles. Well-developed chloroplasts were elliptical in shape with large starch grains occupying the most of the chloroplast volume (Figs. 31, 32). The lamellar system consisted of well-developed grana and intergrana (Figs. 31, 32). Mitochondria were predominantly spherical to elongate in outline (Fig. 32). The nuclei were generally large and elongated (Fig. 31) and possessed a condensed nucleolus (Fig. 32).

In dwarf mangrove leaves, palisade cells showed a distinct withdrawal of the plasma membrane and cytoplasm from the cell wall and a characteristic deformation and disintegration of the integrity of chloroplasts. In addition, there were a number of multivesicular structures associated within the palisade cells and small vesicular structures enclosed within larger vesicles were visible in the cytoplasm and the vacuoles of palisade cells (Figs. 33, 34). Leaves of fringe mangroves possessed larger starch grains and fewer plastoglobuli (Figs. 31, 32) than those of dwarf mangrove leaves (Fig. 34).

Differences in spongy mesophyll cell ultrastructure between fringe and dwarf mangrove leaves.

In leaves of fringe mangroves, the spongy mesophyll cells were oval-to-spherical in shape, with chloroplasts evenly distributed along the tangential walls of the cell (Fig. 37). The prominent nuclei, enclosed by distinct double membranes, were housed in the cells. Granal stacks and integranal lamellae appeared to be well developed, radiating throughout the chloroplast stroma with large starch grains (Fig. 38). Plasmodesmata interconnected the adjacent cells (Fig. 38). Abundant mitochondria were distributed in the vicinity of the chloroplasts and along the peripheral region of the cell walls (Fig. 38). In leaves of dwarf mangroves, the spongy mesophyll cells appeared to be asymmetrical in shape, the nucleus lacked a distinctive double nuclear envelope and the nucleolus appeared to have degenerated (Fig. 39, white arrows). The plasma membrane

PLATE 9

Figures 37-38. Ultrastructural detail of spongy mesophyll cells in leaves of fringe mangroves.

Fig. 37. Abundant, uniformly distributed chloroplasts (short arrows) occupying the peripheral regions of loosely arranged spongy mesophyll cells. Note vacuoles (Va) of varying sizes, large nuclei (N) occupying central positions of cell. Lower epidermis (LE) covered by thin cuticle (c). Stoma and associated guard cells showing prominent nucleolus (nu) in large nucleus (N) centrally located in cytoplasm that was rich with abundant ribosomes (arrow).

Bar = 2 μ m

Fig. 38. Cytoplasm contains mitochondria (short arrows) associated with well-developed chloroplasts (Cl). Large nucleus (N) occupying more or less central portion of cell. Note adjacent cells interconnected symplastically by plasmodesmata (arrow).

Bar = 2 μ m

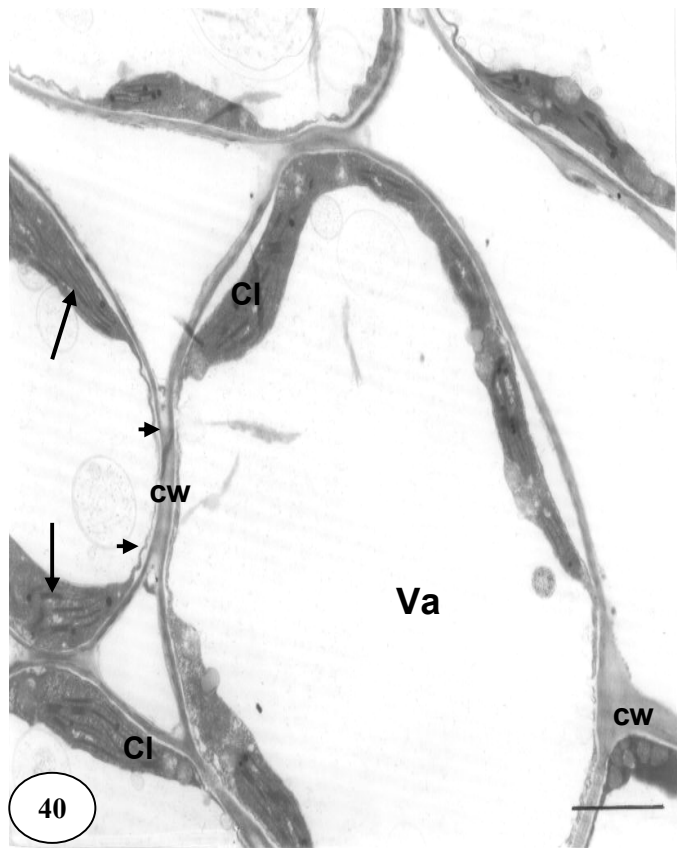
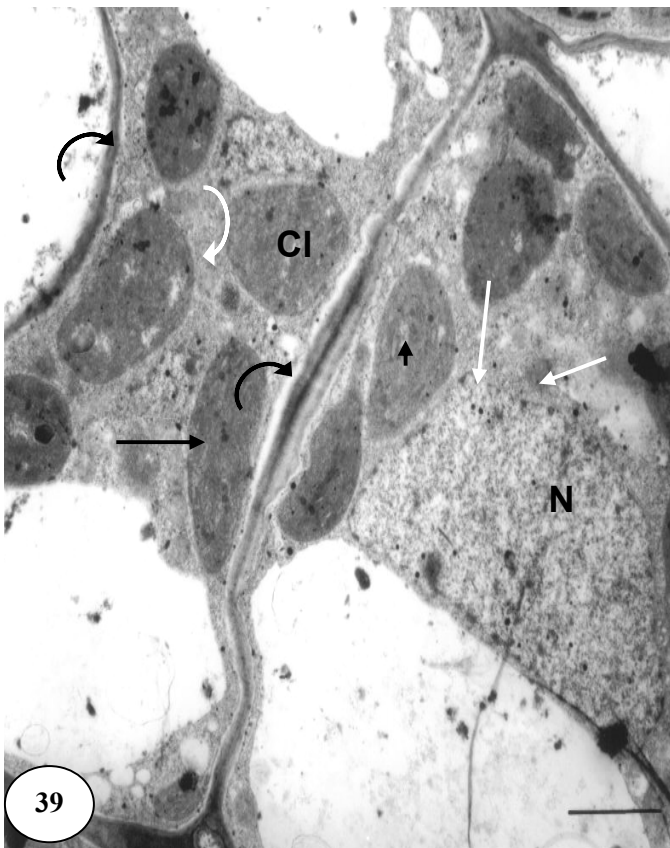
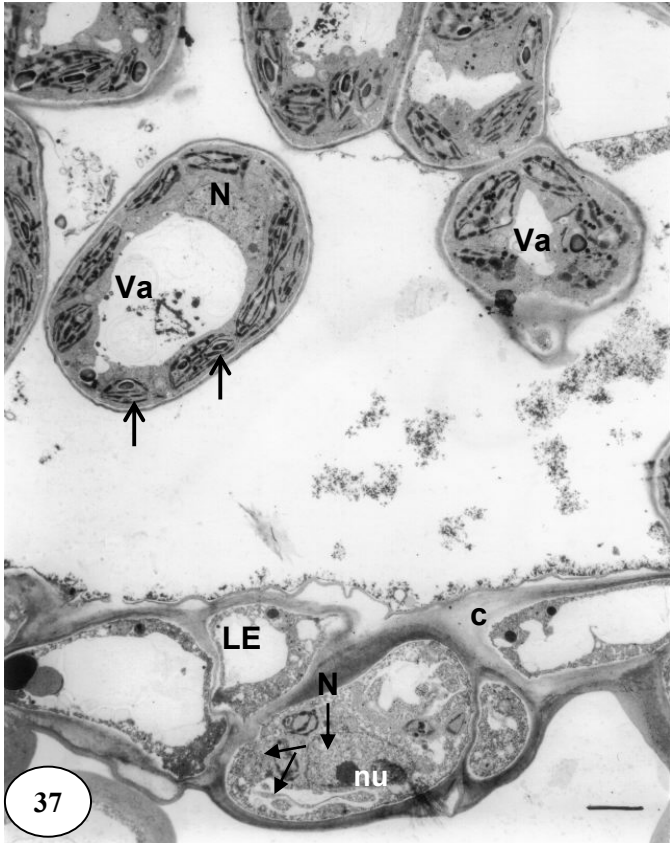
Figures 39-40. TEM of spongy mesophyll cells in leaves of dwarf mangroves.

Fig. 39. Note disorganisation of chloroplast structure: disintegration of chloroplast envelope (arrow), granal stacking and integranal lamellae indistinguishable (arrowheads), chloroplasts (Cl) appeared to be highly swollen and displaced from peripheral cell boundary (curved arrows). Nucleus (N) was centrally located with disintegrating nuclear membrane and nucleolus (white arrows).

Bar = 2 μ m

Fig. 40. Chloroplasts (Cl) (arrows) restricted to cell periphery. Withdrawal of plasma membrane (arrowhead) from cell wall was evident. Cells appeared to be highly vacuolated (Va).

Bar = 2 μ m



and surrounding cytoplasm were withdrawn from the cell wall (arrowheads). The cell walls appeared to be thickened, wavy and irregular in outline (Fig. 40).

Cytoplasm

In leaves of fringe mangroves, the cytoplasm constituted a thin parietal layer, closely appressed to the wall by a large central vacuole which occupied the bulk of the cell volume (Fig. 31). In dwarf mangrove leaves, where plasmolysis occurred, the cytoplasm and organelles appeared to be withdrawn from the cell wall (Fig. 40).

Nucleus

In leaves of fringe mangroves, the nucleus was generally large and spherical with a distinct nuclear envelope, and possessed a condensed nucleolus (Figs. 31, 32). The overall density of the nucleoplasm was similar to that of the cytoplasm. The nuclei of these cells had a general homogenous nucleoplasm with a density similar to that of the surrounding cytoplasm. However, in leaves of dwarf mangroves the nuclei appeared to be distorted causing the double nuclear envelope to appear fragmented (Figs. 36, 39).

Chloroplasts

Chloroplasts of fringe mangrove leaves appeared to be more ellipsoidal or lens-shaped with a clear inner structure (Figs. 31, 41, 42) while those in dwarf mangrove leaves appeared to be dilated and distorted (Figs. 33, 34, 35). The internal membrane system consisting of stacked grana connected by intergranal thylakoids appeared to be well developed. In addition, smaller plastoglobuli were found in leaf chloroplasts of fringe mangroves (Fig. 42). Chloroplasts of fringe mangrove leaves were usually arranged with their shorter axes orientated normal to the plane of the adjacent cell surfaces with relatively large starch grains which comprised approximately 10 – 30 % of the plastid contents (Figs. 31, 32). The grana, which occupied approximately one-third of the total plastid volume, consisted of many layers of thylakoids stacked in an orderly manner (Fig. 41).

There were distinct differences in leaf chloroplast morphology between fringe and dwarf mangrove leaves. Chloroplasts in leaves of dwarf mangroves had swollen, dilated thylakoids (Fig. 35, 47), no membrane integrity (Fig. 35), disintegrated granal stacks and intergranal lamellae (Figs. 47, 48), as well as displacement of the chloroplast membrane from the plasma membrane

PLATE 10

Figures 41-42. Ultrastructural detail of palisade and spongy mesophyll chloroplasts in leaves of fringe mangroves.

Fig. 41. Palisade mesophyll chloroplast housing large starch grain (sg) occupying most of the chloroplast stroma. Thylakoids (long arrows) closely appressed forming typical stack-like grana (g) interconnected by fairly straight and closely appressed intergranal lamellae (short arrows).

Bar = 200 nm

Fig. 42. Spongy mesophyll chloroplast enclosed by intact double-membrane (arrows). Granal stacks (g) well defined and radiating continuously throughout the plastid. Plastoglobuli (p) present between intergranal lamellae (short arrows).

Bar = 500 nm

Figures 43-46. TEM showing palisade and spongy mesophyll chloroplasts in leaves of dwarf mangroves.

Fig. 43. Palisade mesophyll chloroplast with large irregularly shaped centrally situated starch grain (sg). Chloroplast envelope appeared indistinct (arrowheads). Granal stacks (g) and intergranal lamellae (short arrows) appear disorganised, thylakoids not closely appressed to each other.

Bar = 200 nm

Fig. 44. Disorganised chloroplast of spongy mesophyll cell showing unusually large plastoglobuli (p).

Bar = 100 nm

Fig. 45. Separation of chloroplast granal stacks (arrowheads), thylakoids and intergranal lamellae (arrows) appeared abnormally wavy and not closely appressed in spongy mesophyll cell.

Bar = 200 nm

Fig. 46. Higher magnification of disintegrating granal stack. Note separation of thylakoid lamellae (arrows)

Bar = 100 nm

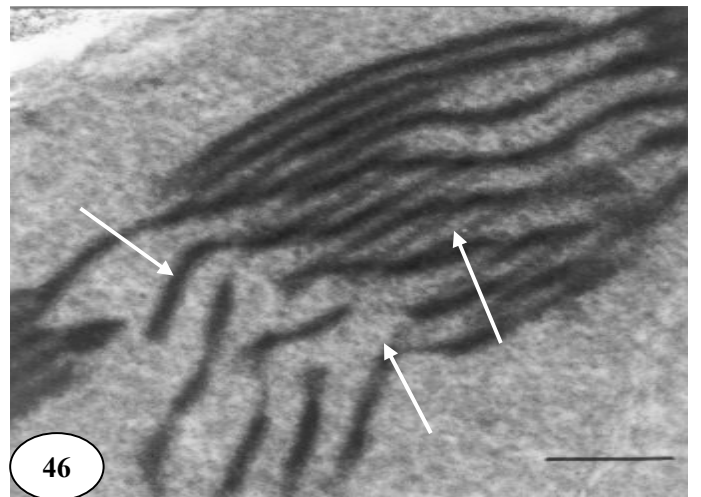
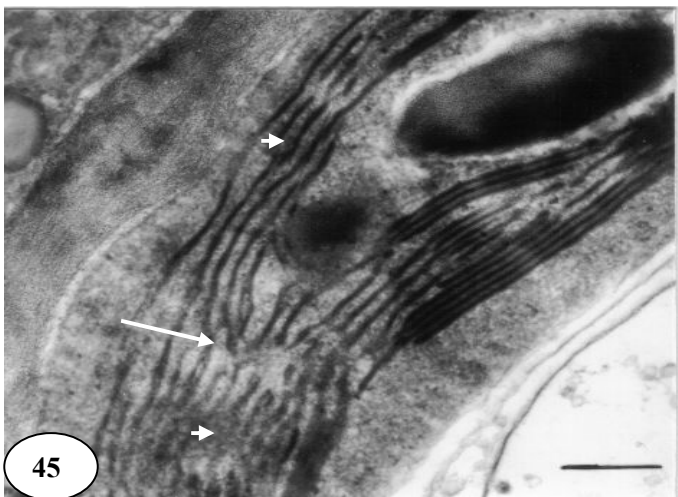
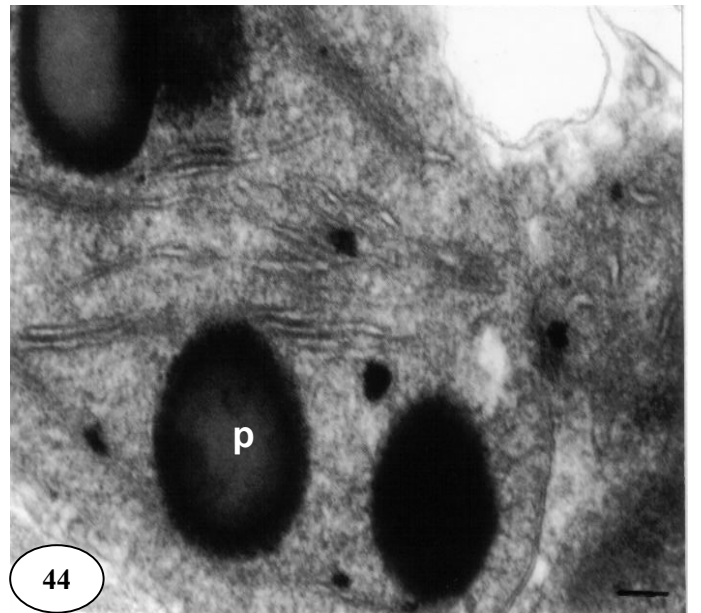
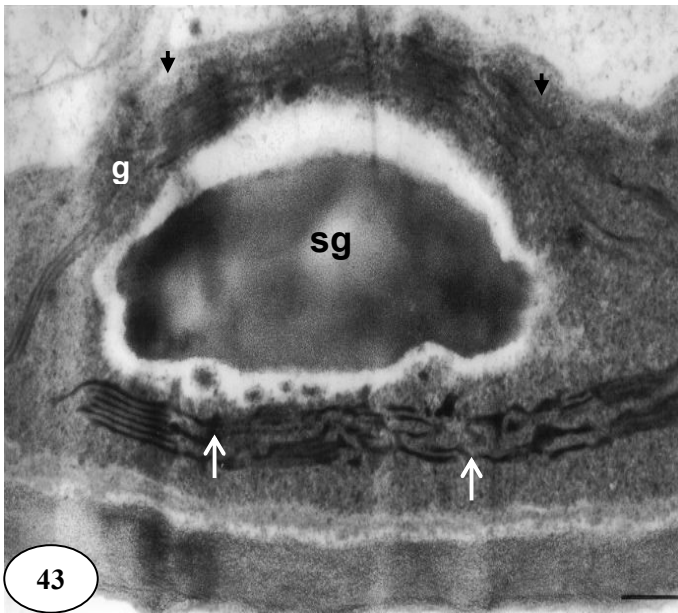
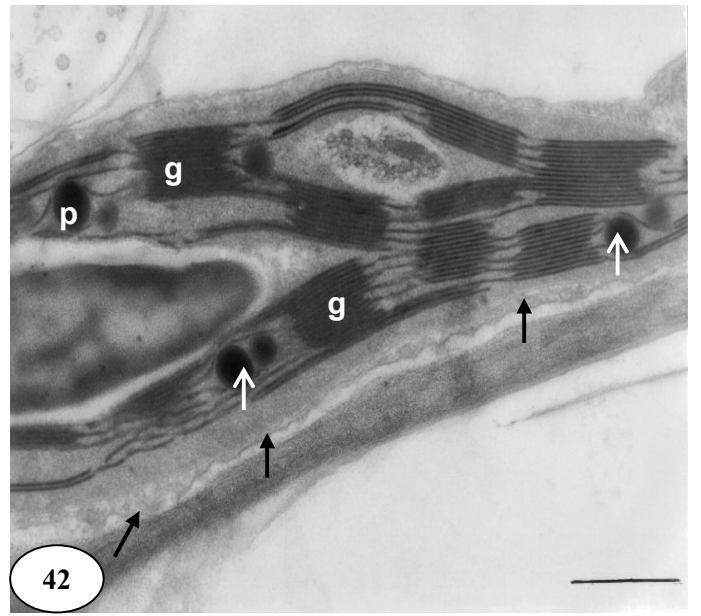
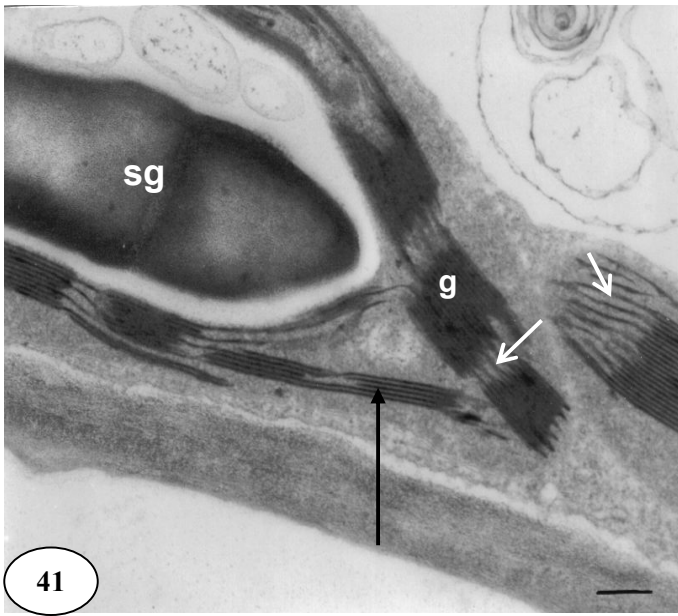


PLATE 11

Figure 47. Ultrastructural detail of spongy mesophyll chloroplasts in leaves of dwarf mangroves showing complete breakdown of lamellae system. Note dilated thylakoid membranes (arrowheads) and plasma membrane completely displaced from cell wall (arrows).

Bar = 200 nm

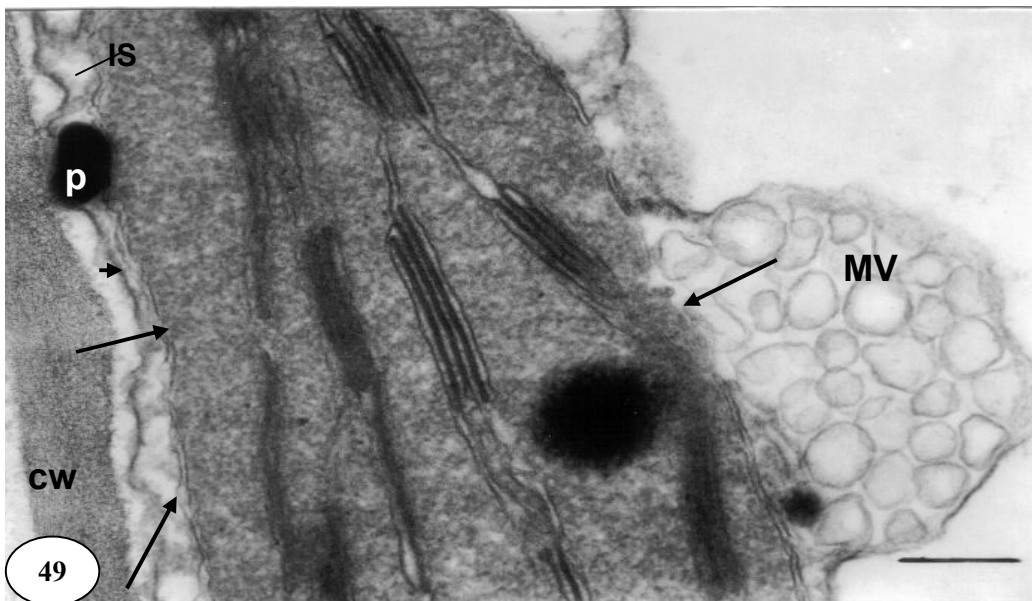
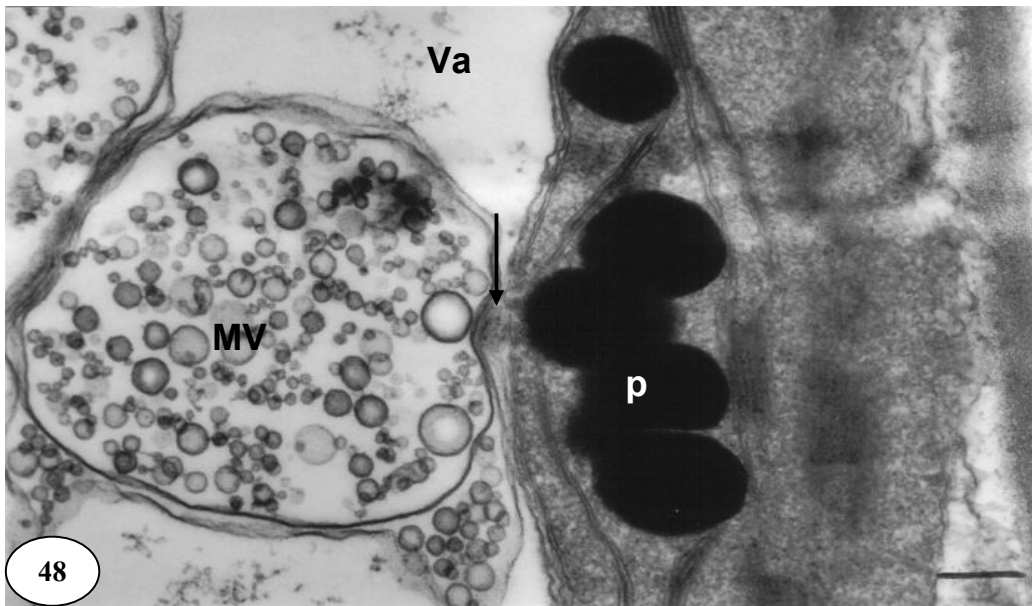
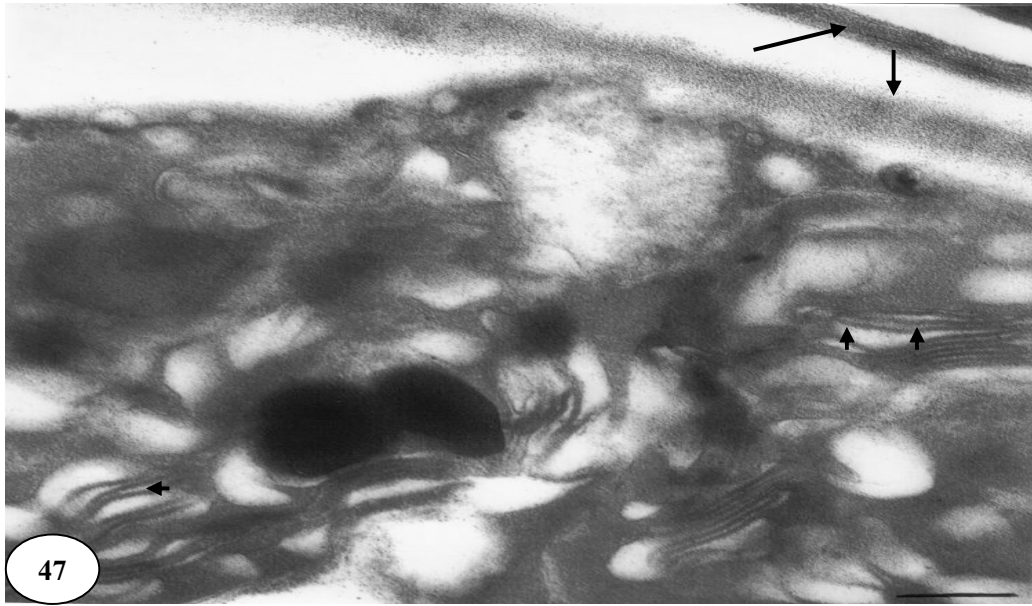
Figures 48-49. TEM of multivesicular structures in spongy mesophyll cells in leaves of dwarf mangroves.

Fig. 48. Multivesicular structures (MV) found within the vacuole (Va) in close proximity to chloroplast envelope (arrow). Note unusually large plastoglobuli (p) in chloroplast.

Bar = 200 nm

Fig. 49. Multivesicular structure (MV) in close association with chloroplast envelope (arrow). Large plastoglobuli (p) appear to be moving out of chloroplast stroma into intracellular space (IS). Plasma membrane retracted from cell wall (arrowhead).

Bar = 200 nm



boundary (Fig. 49). Chloroplast profiles showed a striking arrangement around the nucleus. Although their shape was basically similar to leaves of fringe mangroves, chloroplasts in leaves of dwarf mangroves exhibited amoeboid deformations (Figs. 35, 36). Several large plastoglobuli appeared clustered within the granular stroma of chloroplasts and nucleoid areas (Fig. 44). In leaves of dwarf mangroves, plastoglobuli accumulated in the chloroplasts and sometimes fused with the chloroplast envelope and appeared to empty their contents into the cytoplasm (Fig. 49).

Vacuoles

Many forms of membranous structures were encountered within the vacuoles of dwarf mangrove leaves (Figs. 34, 35). Vacuoles were irregular in shape (Figs. 39, 40). In both leaf types, vacuolar contents consisted mainly of dispersed fibrillar-like material in addition to a number of membranous structures (Figs. 34, 38). Vacuoles of fringe mangrove leaves were large and of varying sizes, due to fusion (Fig. 37). Some vacuoles of dwarf mangrove leaves appeared to be engulfing myelin-like membranous configurations (Fig. 51) and also small vesicular structures within larger vesicles (Figs. 34, 48, 49). In dwarf mangrove leaves, cytoplasmic and vacuolar contents appeared disorganised (Figs. 34, 50).

Cell wall and plasma membrane

In fringe mangrove leaves, the plasma membrane was closely appressed to the cell walls (Figs. 31, 33), whereas in leaves of dwarf mangroves the plasma membrane was generally more irregular and showed a distinct withdrawal from the walls (Figs. 33, 36, 40). This phenomenon was most pronounced along the outer tangential walls where large extracytoplasmic spaces (formed by the withdrawal of the plasma membrane from the walls) occurred (Fig. 33). A large number of plasmalemmasomes (vesicles associated with the plasma membrane) and other membranous structures were observed in the extracytoplasmic spaces of dwarf mangrove leaves (Figs. 33-thin arrows, 53-long arrows).

The irregularity in the outline of the plasma membrane was more extensive in dwarf mangrove leaves (Fig. 33). Undulations of the plasma membrane were pronounced along the inner tangential walls and also extended along the radial walls (Fig. 33). In these regions, in the irregular extracytoplasmic space, small, multivesicular structures were present (Fig. 53). The morphology of these structures closely resembled that of the plasmalemmasomes (Fig. 33).

The outline of the plasma membrane in dwarf mangrove leaves was extremely asymmetrical and generally showed greatest withdrawal from the walls, thus forming an undulating extracytoplasmic space. In the vicinity of the plasmodesmata, the cell walls were generally thickened (Fig. 53). Ultrastructural features associated with dwarf mangrove leaves included increased withdrawal of the plasma membrane and cytoplasm from the cell wall, breakdown of the cell wall, increased vacuolation, and changes in the morphology of mitochondria and plastids (Fig. 51).

Mitochondria

In leaves of fringe mangroves, mitochondria possessed a well-developed system of prominent cristae and an electron-translucent matrix with electron-dense mitochondrial granules visible in their stroma. Mitochondria occurred singly, or in pairs, less often in groups of three or more (Figs. 54, 55) and were fairly evenly distributed in the cytoplasm. The majority of the mitochondria and other organelles were localised in the cytoplasm near the inner tangential cell wall (Fig. 54). Mitochondrial shape varied from spherical to elongate (Fig. 56). The structural integrity of mitochondria was retained by the presence of intact double membrane envelopes (Fig. 55, arrowheads). However, in leaves of dwarf mangroves the mitochondrial envelope showed signs of disruption or degeneration (Fig. 59). The latter appeared to be evident as a ballooning effect of the outer membrane of the mitochondrial envelope (Fig. 57). This was accompanied by the progressive disappearance of cristae and the matrix (Figs. 56, 57). In addition to the mitochondrial degeneration, cristae were fragmented, swollen and dispersed (Figs. 56, 57) and the matrix appeared electron-transparent (Fig. 36, curved arrows).

Multivesicular structures associated with the cell wall

A common feature noted in dwarf mangrove leaves was the presence of a few to many membranous vesicles in the extracytoplasmic space along the outer peripheral wall (Fig. 52). These vesicles, which resembled the morphology of those aligned along the wall (Fig. 33), were observed in close proximity to the chloroplasts. Small vesicular structures within larger vesicles were visible in the cytoplasm of a few cells (Figs. 48, 49, 51). Various forms of vesicular material, much of which was enclosed by a membrane, occurred within the vacuole, the extracytoplasmic space, and in the cytoplasm. Multivesicular structures were found in vacuoles and were frequently associated with chloroplasts and mitochondria in dwarf mangrove leaves and less abundant in those of fringe mangroves (Figs. 34, 49).

PLATE 12

Figures 50-51. Ultrastructural detail of chloroplasts in leaves of dwarf mangroves.

Fig. 50. Large spherical plastoglobuli (p) prevalent within the chloroplast. Chloroplast envelope (arrowhead) and mitochondrial membranes not clearly defined or fragmented (short arrow). Integranal lamellae and granal stacking appeared to be sparse (long arrows).

Bar = 200 nm

Fig. 51. Large multivesicular structure (MV) commonly found within the vacuole (Va). Note plasma membrane (arrowhead) appeared to be convoluted or infolded and withdrawn from cell wall. It seemed to be a breakdown of the cellulosic nature of the cell wall (arrows).

Bar = 500 nm

Figures 52-53. Ultrastructural detail of cell walls and cytoplasm of adjacent cells in leaves of dwarf mangroves.

Fig. 52. Cell wall (CW) distorted and wavy associated with numerous multivesicular structures (MV).

Bar = 500 nm

Fig. 53. Thick cell wall (CW) interconnected by plasmodesmata to adjacent cells (arrowheads). Chloroplast membrane (arrows) appeared to be disintegrated, granal stacks and thylakoids also appeared to be indistinct (curved arrows). Mitochondria (M) showed dilated cristae (cr). Note multivesicular structures in extracytoplasmic space between wall and membrane (long arrows).

Bar = 200 nm

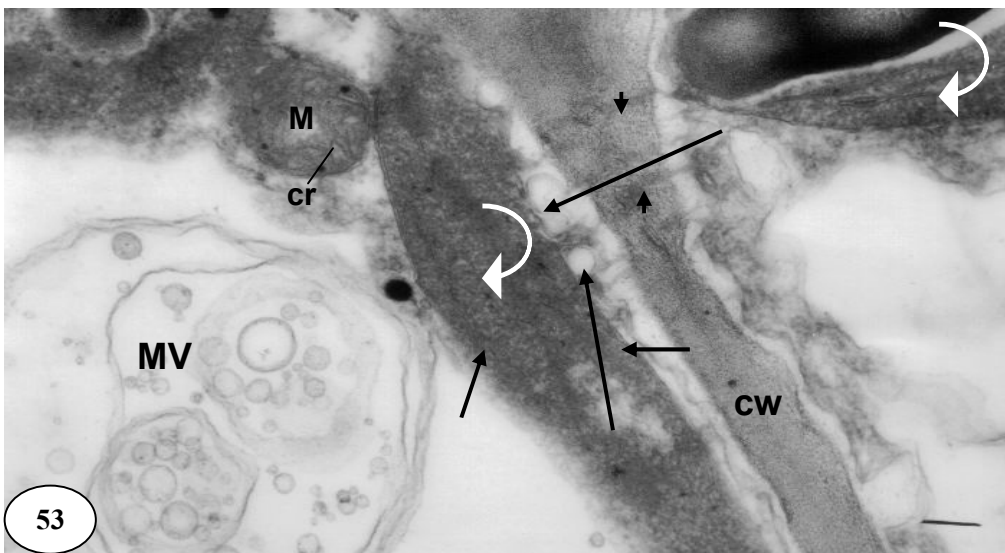
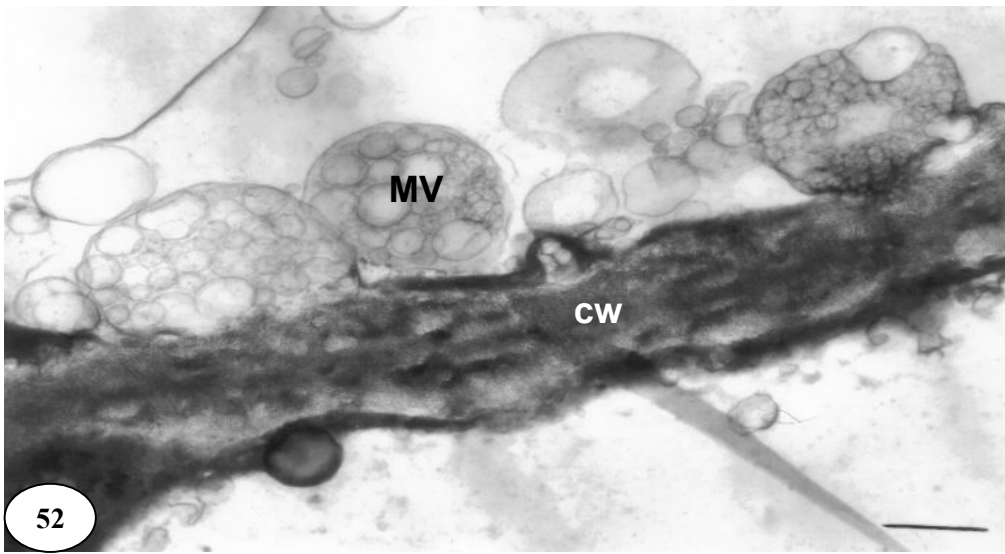
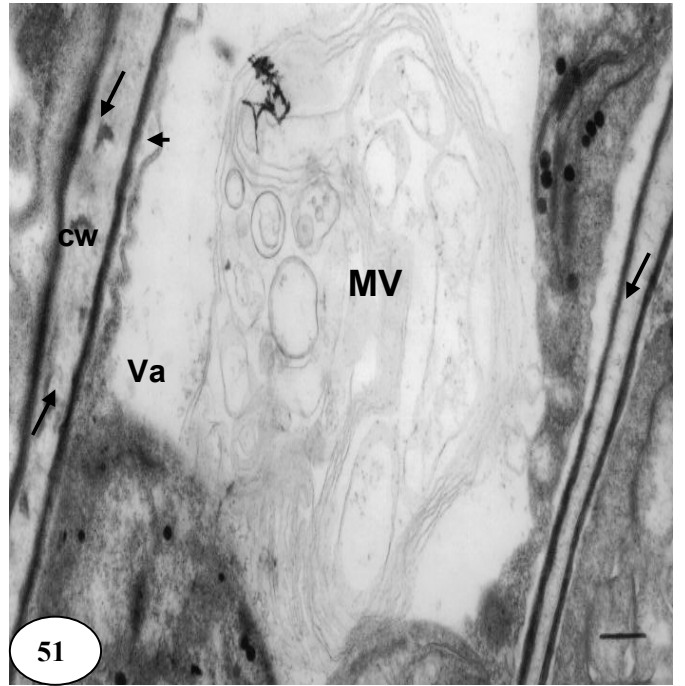
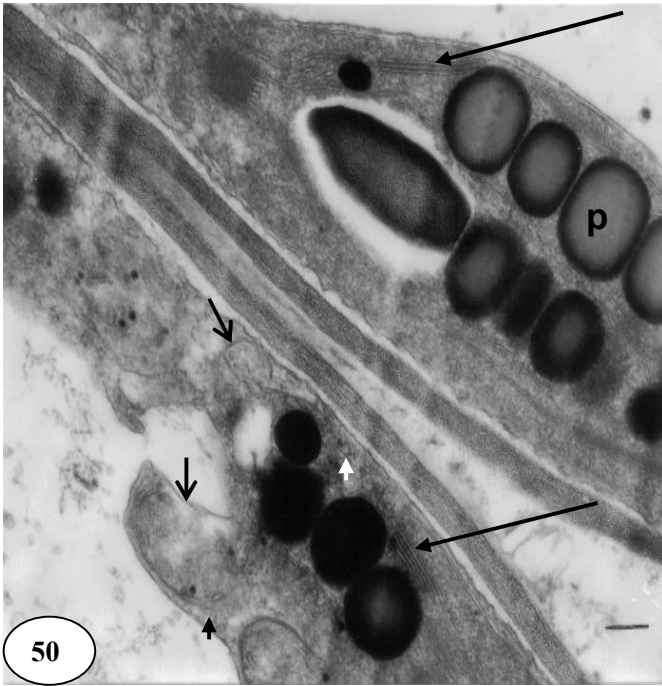


PLATE 13

Figures 54-55. Ultrastructure of mitochondria in leaves of fringe mangroves.

Fig. 54. Typically round to oval mitochondria (M) in palisade mesophyll as seen in cross section. Note the electron-opaque granules found within the mitochondria (arrows).

Bar = 200 nm

Fig. 55. Mitochondria (M) in spongy mesophyll cells with well-developed double membrane (arrowheads) and abundant cristae (short arrows).

Bar = 100 nm

Figures 56-57. Ultrastructural details of mitochondria in palisade and spongy mesophyll in leaves of dwarf mangroves.

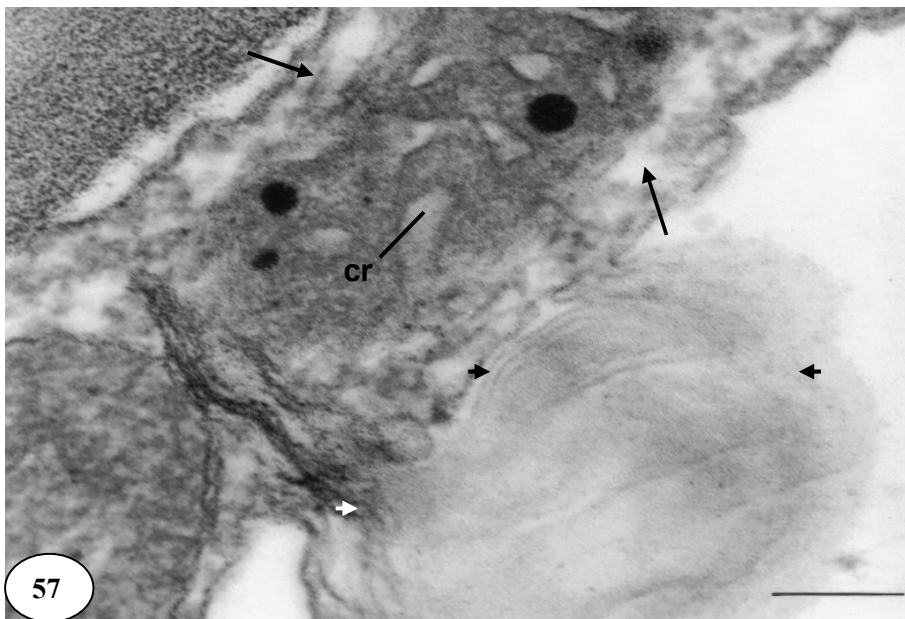
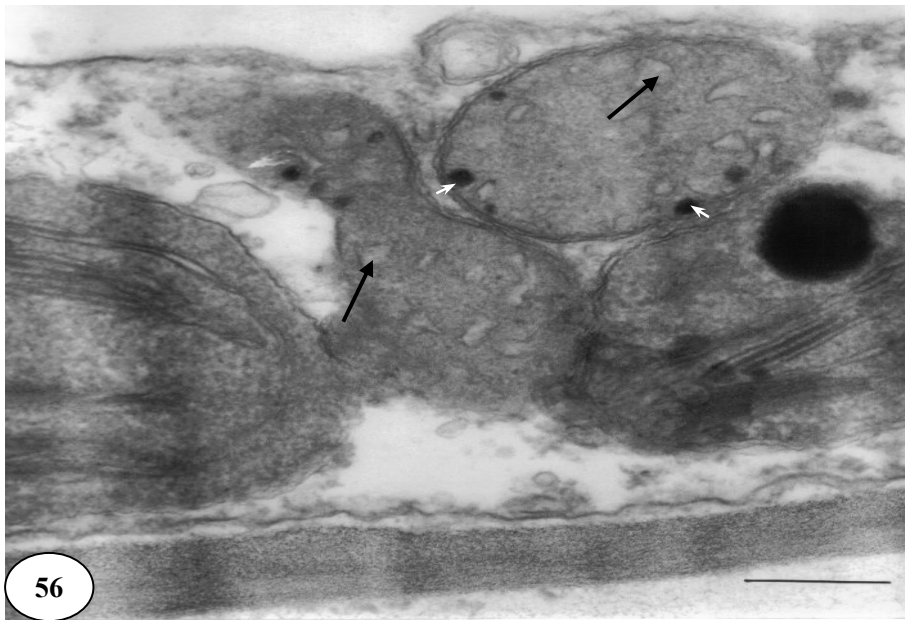
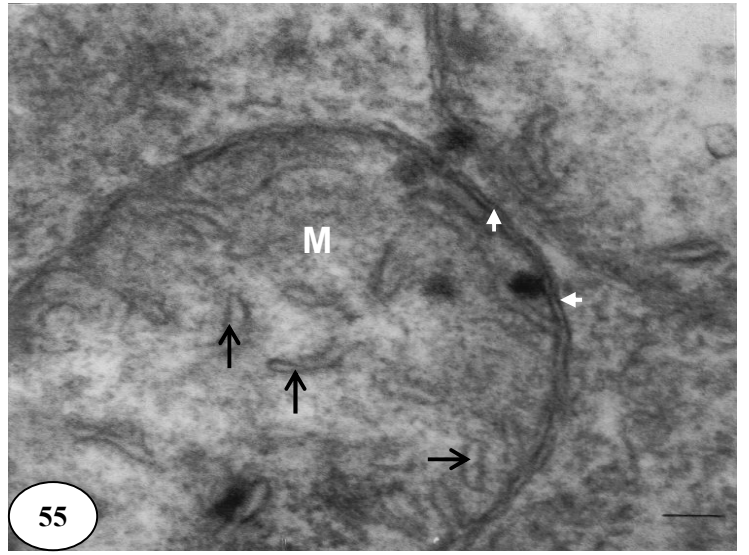
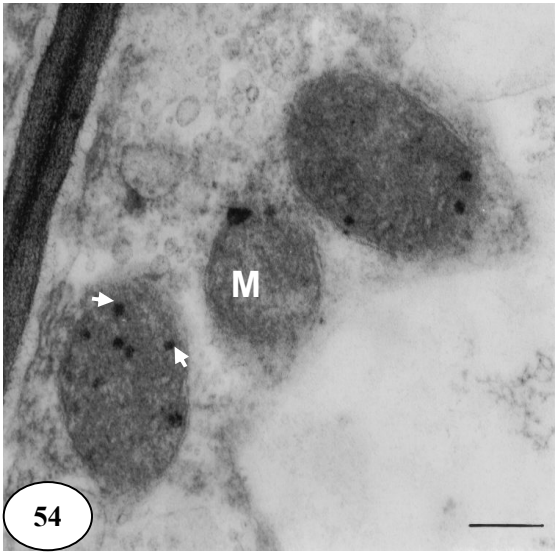
Fig. 56. Round to oval to dumb-bell shaped mitochondria were seen showing dilated cristae (arrows). Dark globular structures (arrowheads) in peripheral region of mitochondrial stroma were probably lipid.

Bar = 200 nm

Fig. 57. Disintegration of mitochondrial membrane system. Note swelling and vacuolation of cristae (cr) and membranes (arrows). The double membrane envelope appeared to be indistinguishable. The mitochondrial stroma appear to be sequestered into the vacuole (arrowheads).

Bar = 100 nm

56



Salt glands and trichomes

Generally, the salt glands of both fringe and dwarf mangrove leaves consisted of 2 to 4 basal collecting cells, one or two disk-like cutinised stalk cells and 8 (sometimes 12) radially arranged secretory or excretory cells, which were covered with a thin cuticle (Figs. 58, 59). The abaxial gland, like the adaxial one was multicellular (Figs. 58, 59). A thick layer of amorphous material was present between the cuticle and the upper walls of the secretory cells of the glands (Fig. 59, curved arrow). Vesicles of varying sizes were commonly observed in the secretory, stalk and collecting cells (Figs. 58, 59). The cytoplasm was rich in organelles such as ER, Golgi bodies and mitochondria, which collectively occupied a major portion of the cell volume (Fig. 58). The vacuoles were smaller in secretory cells of fringe and larger in those of dwarf mangrove leaves. The cytoplasm in leaves of fringe mangroves appeared to be retracted in one of the secretory cells, and the region between it, and the cell wall was filled with an amorphous electron-dense matter (Fig. 58). Within the cytoplasm of secretory cells there were elongated membranes in the vicinity of the nucleus and vacuoles (Fig. 58). These elongated, narrow vacuole-like structures with tapered ends were observed in secretory cells but were lacking in stalk cells (Fig. 58). In these “vacuole-like structures” many “electron-dense areas” with electron-dense margins were observed in secretory cells (Figs. 58). Many of the structures enclosed fibrillar-like material (Figs. 58, 59). The nuclei were large, several mitochondria were clustered while others were evenly dispersed in the cytoplasm of the secretory cells (Fig. 58).

In dwarf mangrove leaves, vacuoles in the secretory cells of the abaxial glands were numerous and larger than those of fringe mangrove leaves (Fig. 59). Mitochondria were scattered in the cytoplasm (Fig. 59). Multivesicular structures featured in secretory, stalk and collecting cells of glands in both dwarf and fringe mangrove leaves. Many multivesicular structures were found within the vacuoles and also between the cytoplasm and the cell wall (Fig. 59). The cytoplasm of the second or third secretory cells in figures 58 and 59 was withdrawn from the thickened cell walls suggesting plasmolysis. However, upon further glandular ultrastructural review in both leaf types, plasmolysis appeared to be more prevalent in glands of dwarf than those of fringe mangrove leaves.

In both fringe and dwarf mangrove leaves, secretory and stalk cells had relatively large nuclei (Figs. 58, 59). The side walls of the stalk cell appeared to be heavily cutinised (Fig. 58, 59) and dark as evident in the light micrograph (Fig. 60). As shown in Fig. 59 (long arrows), the

PLATE 14

Figure 58. L/S of abaxial leaf salt gland of fringe mangrove. Salt gland shows two large secretory cells (S), a smaller stalk cell (St) and an enlarged collecting cell (Co) embedded in leaf surface below level of epidermis (E). Cuticle (c) appears continuous on walls of epidermal (long arrows), secretory (long arrows) and stalk cells (long arrows) as seen in cross section. Expanded cuticle forming cuticular chamber (CH) above secretory cells. Thin-walled secretory cells comprised of a large centrally located nucleus (N) and small vacuoles (arrowheads) and a cluster of mitochondria (short arrows) in ribosome-rich cytoplasm (Cp). Note elongated vacuole-like membranes within the cytoplasm of secretory cells found near and surrounding the small vacuoles (diamond-head arrow). Stalk cell possessed numerous small vacuoles (Va) referred to as mini-vacuoles. The elongated, narrow vacuole-like structures with tapered ends were absent in stalk cells. Collecting cell has high vacuolar volume (Va) with cytoplasm restricted towards the periphery of cell (short white arrows).

Bar = 2 μm

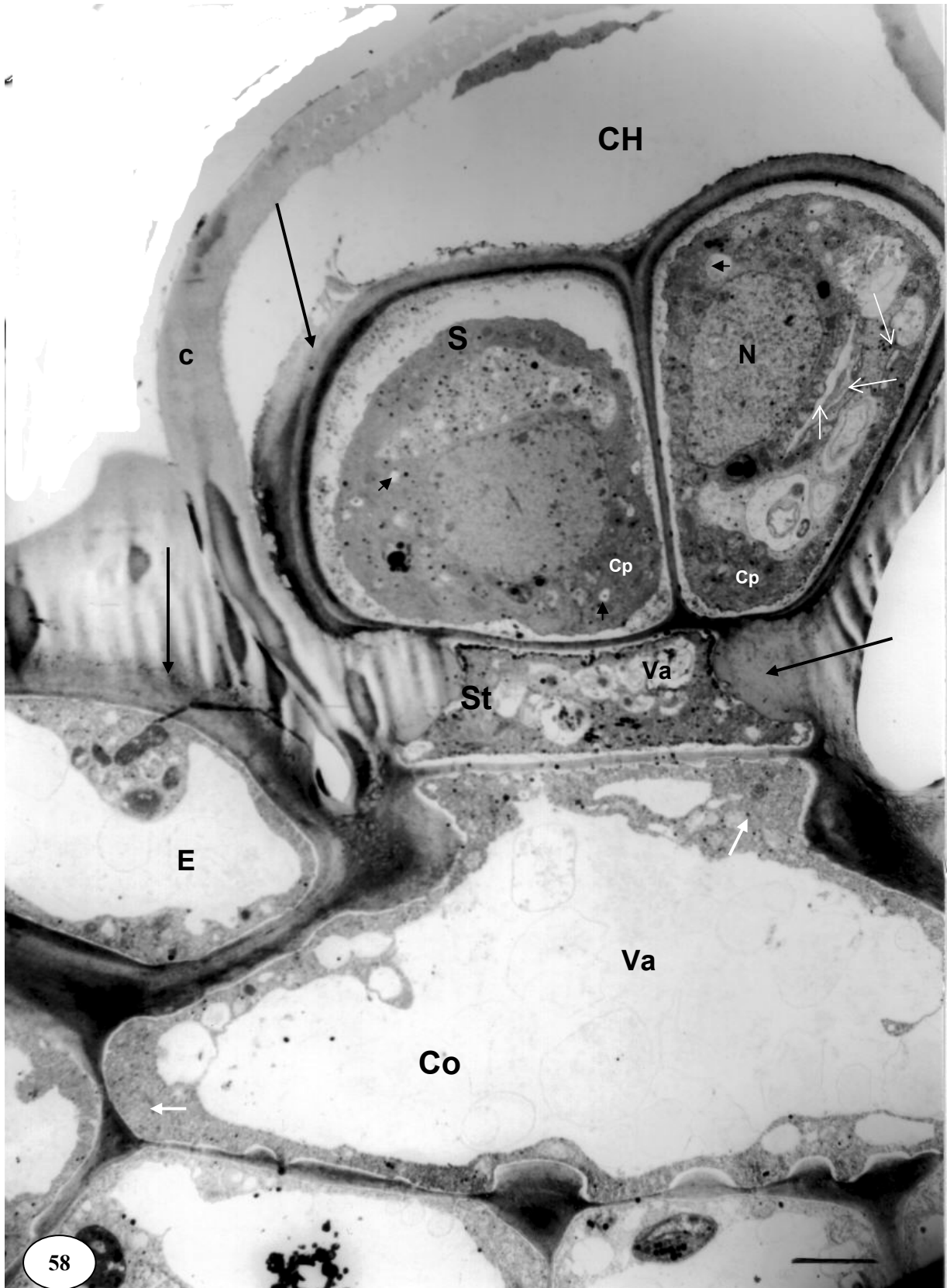


PLATE 15

Figure 59. Ultrastructural detail of distal region of abaxial salt gland in dwarf mangrove leaf showing thick expanded cuticle (c) over cuticular chamber (CH). Cell walls of secretory cells appeared to be thickened (arrows), cell wall of smaller secretory cells form a thick mottled layer (curved arrow). Numerous vacuoles (Va) in secretory cells (S). Mitochondria scattered in secretory cells (arrowheads). Note withdrawal of stalk cell cytoplasm from common wall between secretory and stalk cells (long arrows). Broad stalk cell (St) comprises numerous, mini-vacuoles (Va) and a large centrally situated nucleus (N). Cuticle forming thick layer in transfusion zone between epidermal cell (E) and the constricted side walls (bottle-neck) of stalk cell (thick black arrow).

Bar = 2 μ m



cytoplasm of the stalk cell withdrew from the common horizontal walls of the secretory cells; however, the cutinised side walls of the stalk cells remained intact probably through strong adhesion.

The lower epidermis and the outer tangential walls of trichomes from dwarf mangrove leaves were cutinised (Fig. 60). The trichome was three-celled configured in which the basal cell is heavily cutinised like the adjacent epidermal cells and the terminal cell bears a thin cuticle (Figs. 60, 61).

Stomata

Stomata from fringe mangrove leaves showed no plasmolysis of cytoplasm from cell wall, with fewer plastoglobuli and multivesicular structures (*not shown in results*). In dwarf mangrove leaves, large electron-dense and electron-translucent vacuolar structures were apparent in guard cells of stomata (Fig. 61). Inner tangential walls of stomata appeared highly thickened. The cytoplasm of guard cells was dense with ribosomes, mitochondria, and myelinated structures. The cytoplasm of subsidiary cells was retracted from the cell wall, as if plasmolysed (Fig. 61, arrows). Plastoglobuli appeared as dense aggregates within the degenerating plastids.

PLATE 16

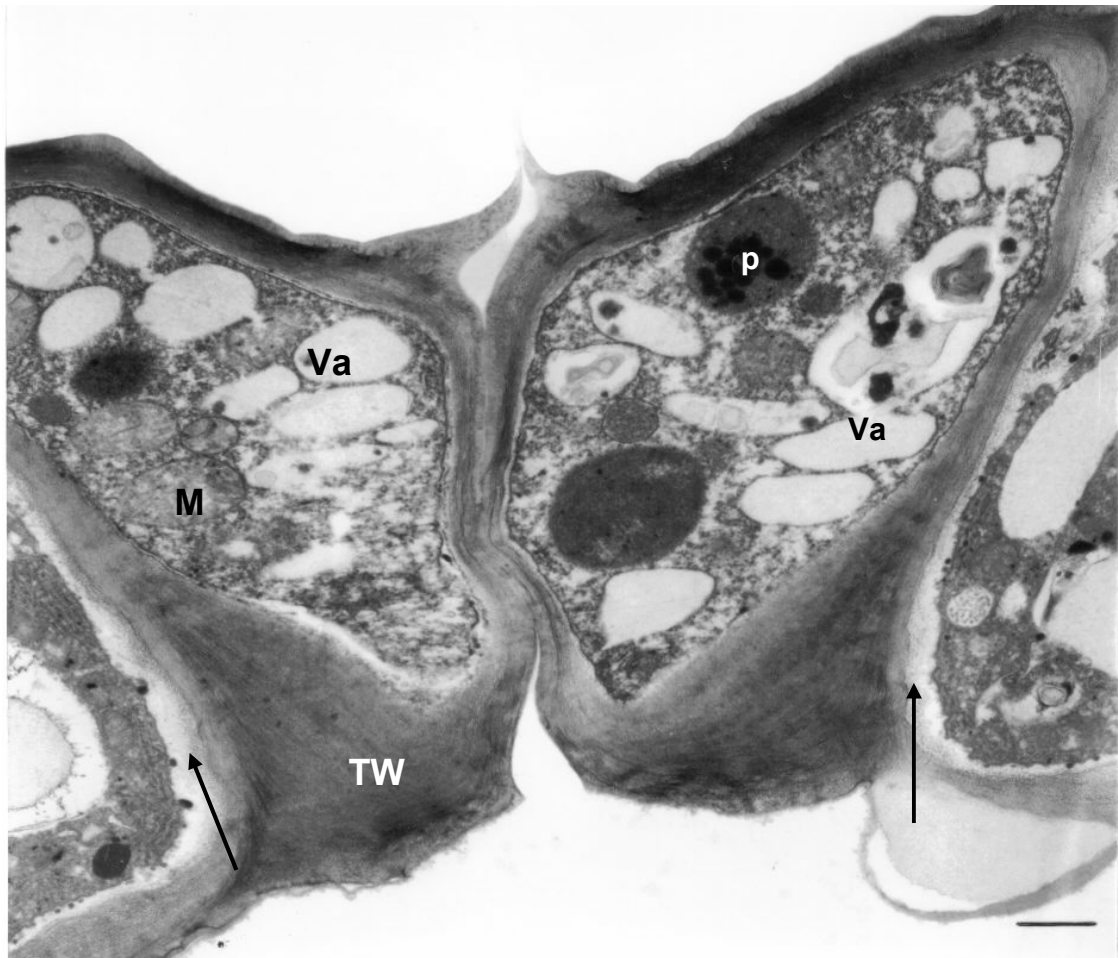
Figures 60-61. LM and TEM showing abaxial stomata of dwarf mangrove leaves.

Fig. 60. Light micrograph showing transverse section of abaxial leaf surface stained with toluidine blue-O. Note thick continuous cuticle (arrows) extending over salt gland (SG) and stoma (st) as well as outer surface of trichomes (t).

Bar = 1.3 μm

Fig. 61. Ultrastructural details of guard cells of abaxial stoma. Note thick extension of inner tangential wall towards the lower region of guard cells (TW). Guard cells had a high vacuolar volume (Va) and abundant mitochondria (M). Plastoglobuli (p) located within the plastids of guard cells. Cytoplasmic content of subsidiary cells appeared to be withdrawn from the neighbouring guard cell walls (arrows).

Bar = 1 μm



4.2. PHYSIOLOGY

Consistent with the dwarfing effect, whole plant performance of *A. marina* generally decreased with increased salinity, from 35 ‰ in fringe mangroves to 60 ‰ in dwarf mangroves. Leaf morphological effects were evident in dwarf mangroves and were characterised by a reduction in specific leaf area and an increase in leaf thickness. Specific leaf area in dwarf mangroves was 17 % lower ($t = 4.16$, $P < 0.001$) than that of fringe mangroves (Fig. 62).

Dwarf mangrove leaves were 27.6 % ($t = 22.64$, $P < 0.0001$) thicker than those of fringe mangroves (Fig. 63). Leaf cuticle was 34.7 % ($t = 6.22$, $P < 0.0001$) thicker in dwarf compared to fringe mangrove leaves (Fig. 64).

Percentage relative water content (% RWC) was significantly lower in leaves of dwarf mangroves by 7.8 % ($t = 1.28$, $P < 0.0001$) compared to those of fringe mangroves (Fig. 65).

The total chlorophyll content, expressed, on a unit fresh weight basis was 27 % lower ($t = 5.62$, $P < 0.0001$) (Fig. 68) in dwarf as compared to those of fringe mangroves.

Similar trends in chlorophylls a and b, expressed on a fresh weight basis, were observed. Both chlorophylls a and b (Figs. 66, 67) were lower by 22 % ($t = 5.49$, $P < 0.0001$) and 39.6 % ($t = 5.57$, $P < 0.0001$), respectively in dwarf mangrove leaves compared to those of fringe mangroves.

Salt gland frequency, which was determined in three regions along the adaxial leaf surface, namely the apex, basal and mid-lamina regions, was higher in the apex, mid-lamina and base of fringe than dwarf mangrove leaves by 36 % ($t = 6.84$, $P < 0.0001$) (Fig. 69), 45 % ($t = 14.6$, $P < 0.0001$) (Fig. 70) and 51 % ($t = 15.75$, $P < 0.0001$) (Fig. 71), respectively. Salt gland frequency was generally higher at the base of the leaves and decreased towards the apex in both fringe and dwarf mangrove leaves.

Osmotic relations: glycinebetaine and proline

HPLC analysis showed significant differences in glycinebetaine content of fringe and dwarf mangrove leaves. The concentration of glycinebetaine was 40 % ($t = 8.29$, $P < 0.0001$) (Fig. 72) higher in dwarf than in fringe mangrove leaves. However, proline concentrations were low but consistently higher in leaves of fringe than in dwarf mangrove leaves by 27 % ($t = 3.37$, $P < 0.05$) (Fig. 73).

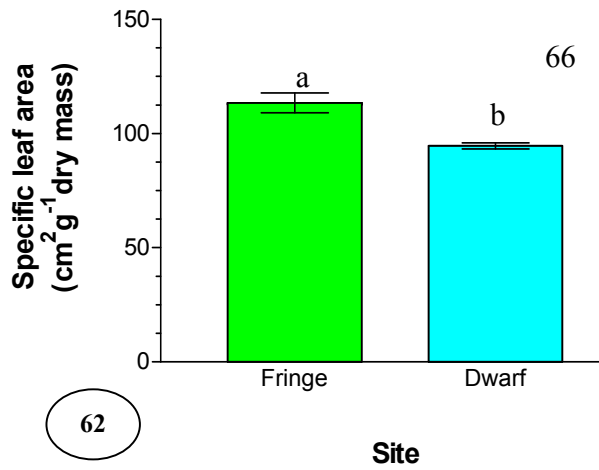


Fig. 62. Specific leaf area (SLA) of leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 9). Bars with different letters are significantly different at $P < 0.001$, using Unpaired t test.

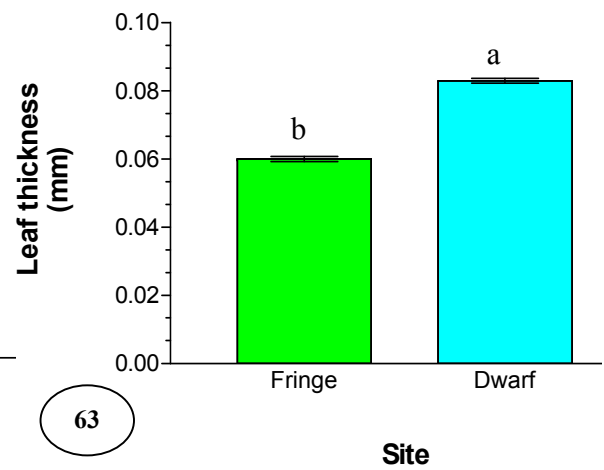


Fig. 63. Leaf thickness of fringe and dwarf mangroves. Values are means \pm SE (n = 170). Bars with different letters are significantly different at $P < 0.0001$, using Unpaired t test.

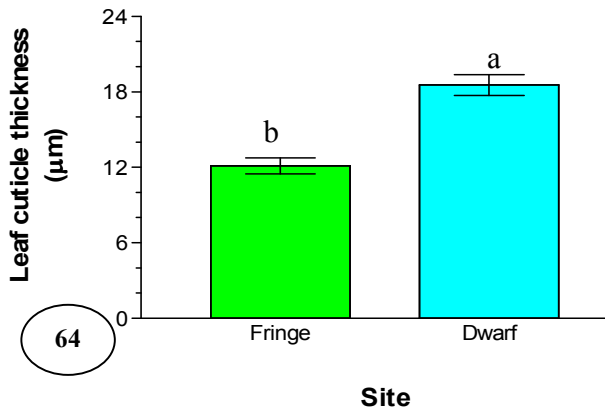


Fig. 64. Leaf cuticle thickness of fringe and dwarf mangroves. Values are means \pm SE (n = 60). Other details as for Fig. 63.

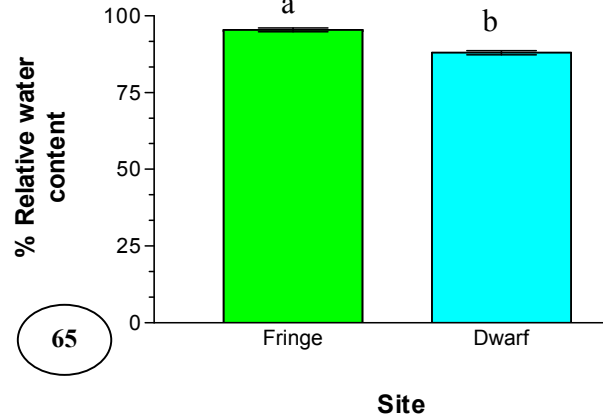


Fig. 65. % Relative water content (RWC) in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 18). Other details as for Fig. 63.

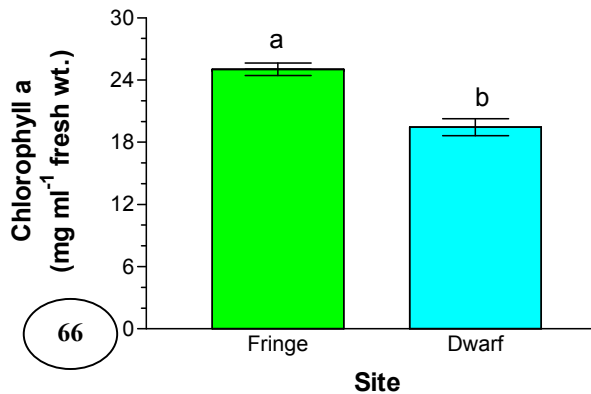


Fig. 66. Chlorophyll a content in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 25). Other details as for Fig. 63.

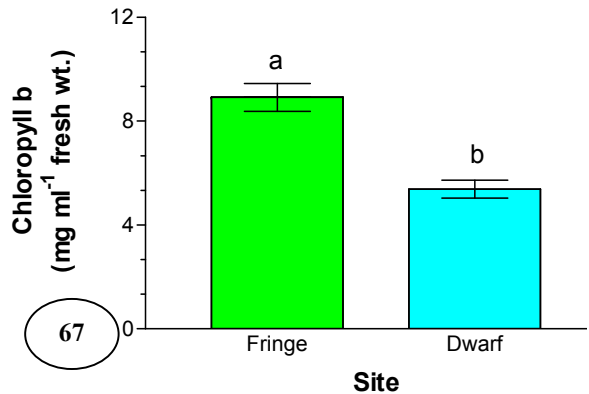


Fig. 67. Chlorophyll b content in leaves of fringe and dwarf mangroves. Other details as for Fig. 66.

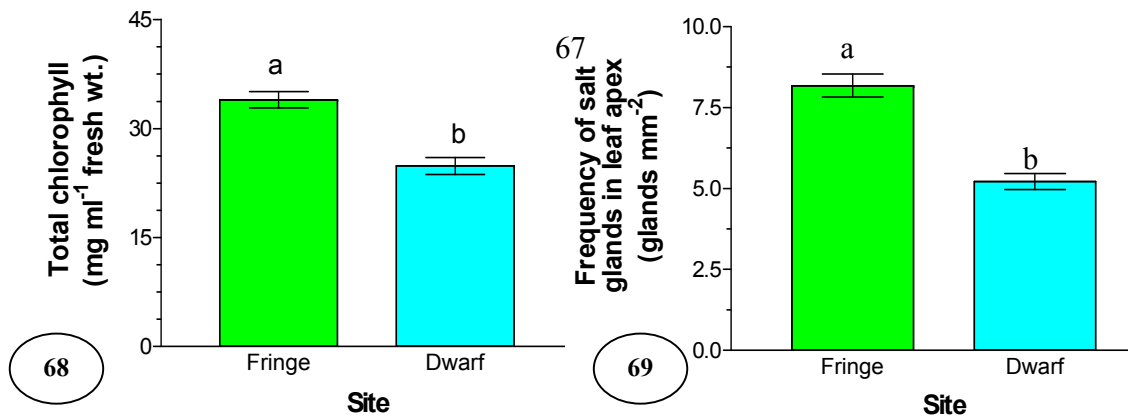


Fig. 68. Total chlorophyll content of leaves from fringe and dwarf mangroves. Other details as for Fig. 66 as before.

Fig. 69. Salt gland frequency at the leaf apex of fringe and dwarf mangroves. Values are means \pm SE (n = 60). Other details as for Fig. 63.

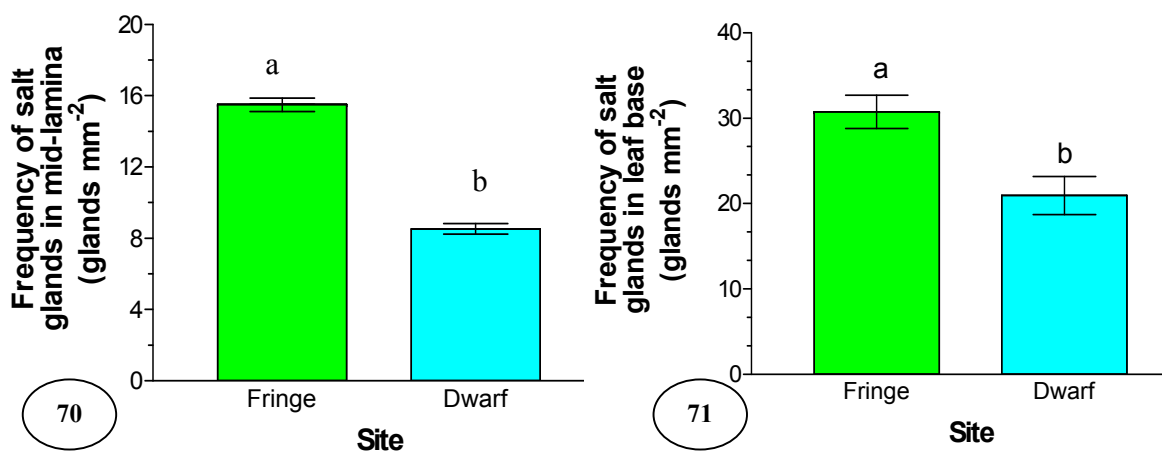


Fig. 70. Salt gland frequency at the mid-lamina region of fringe and dwarf mangroves. Other details as for Fig. 69.

Fig. 71. Salt gland frequency at the leaf base of fringe and dwarf mangroves. Other details as for Fig. 69

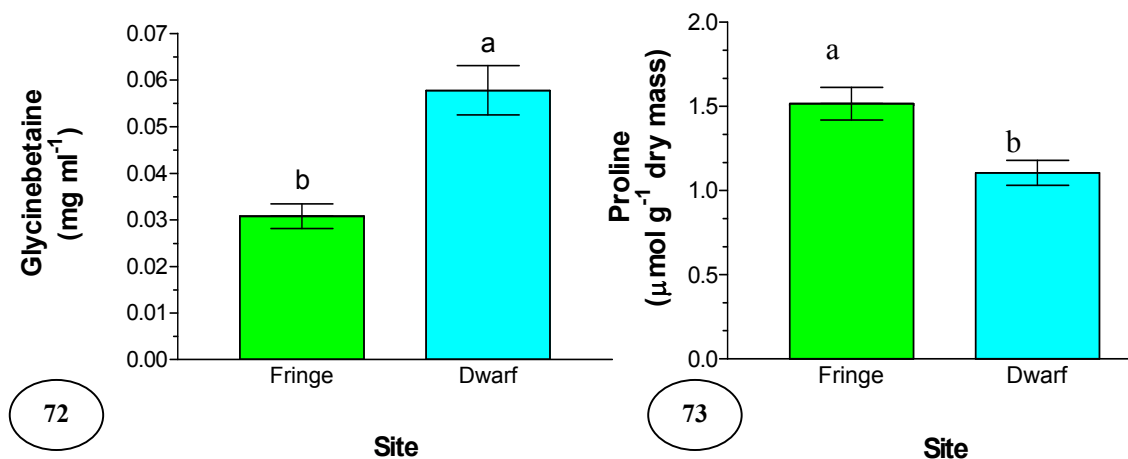


Fig. 72. Glycinebetaine concentrations in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 60). Other details as for Fig. 63.

Fig. 73. Proline concentrations in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 16). Bars with different letters are significantly different at $P < 0.05$, using Unpaired *t* test.

Leaf ion concentrations

Leaf ion concentrations are expressed on a % dry mass basis. The predominant ion in the leaves was Na⁺, concentrations being 19 % ($t = 12.6, P < 0.0001$) higher in dwarf than in fringe mangrove leaves (Fig. 74). In contrast, concentrations of K⁺, Ca²⁺, Mg²⁺ and P were significantly lower in the leaves of dwarf mangroves by 49 % ($t = 11.60, P < 0.0001$) (Fig. 75), 38 % ($t = 10.7, P < 0.0001$) (Fig. 76), 35 % ($t = 3.77, P < 0.001$) (Fig. 77) and 50 % ($t = 12.60, P < 0.0001$) (Fig. 78), respectively. The Na⁺/K⁺ ratio was 62 % greater in dwarf than fringe mangrove leaves ($t = 10.6, P < 0.0001$) (Fig. 79). Nitrogen concentration in leaves of dwarf mangroves was significantly higher than those in the fringe zone by 14 % ($t = 3.36, P < 0.05$) (Fig. 80). There were no significant differences in leaf concentrations of Zn²⁺ (Fig. 81), Cu²⁺ (Fig. 82), and Fe (Fig. 83) between sites, although Mn²⁺ was 27 % ($t = 4.60, P < 0.001$) lower in dwarf than in fringe mangrove leaves (Fig. 84).

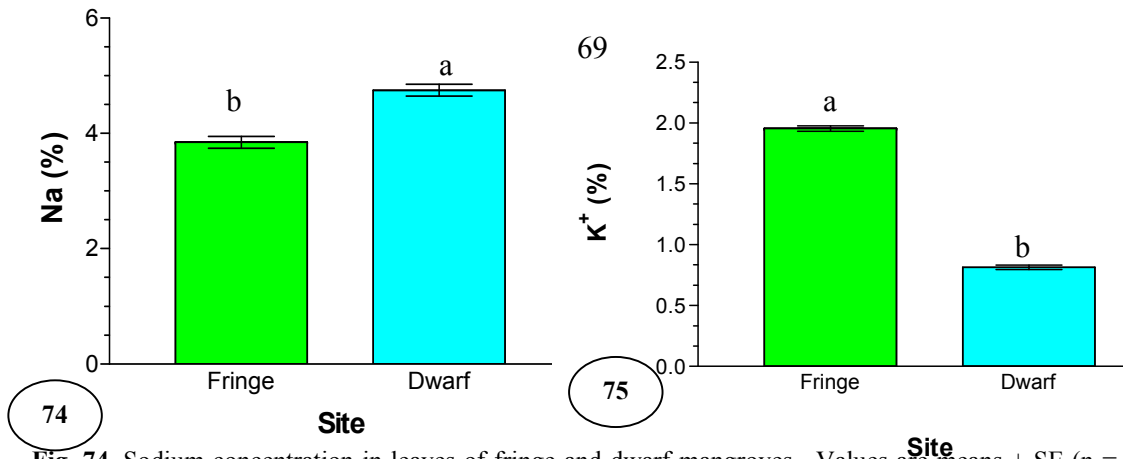


Fig. 74. Sodium concentration in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 6). Other details as for Fig. 63.

Fig. 75. Potassium concentration in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 10). Other details as for Fig. 63.

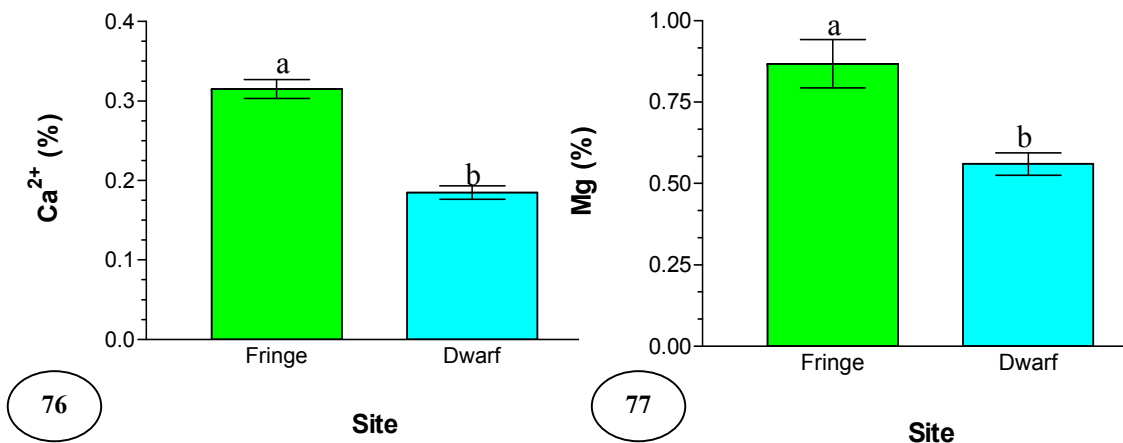


Fig.76. Calcium concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 75.

Fig.77. Magnesium concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 75.

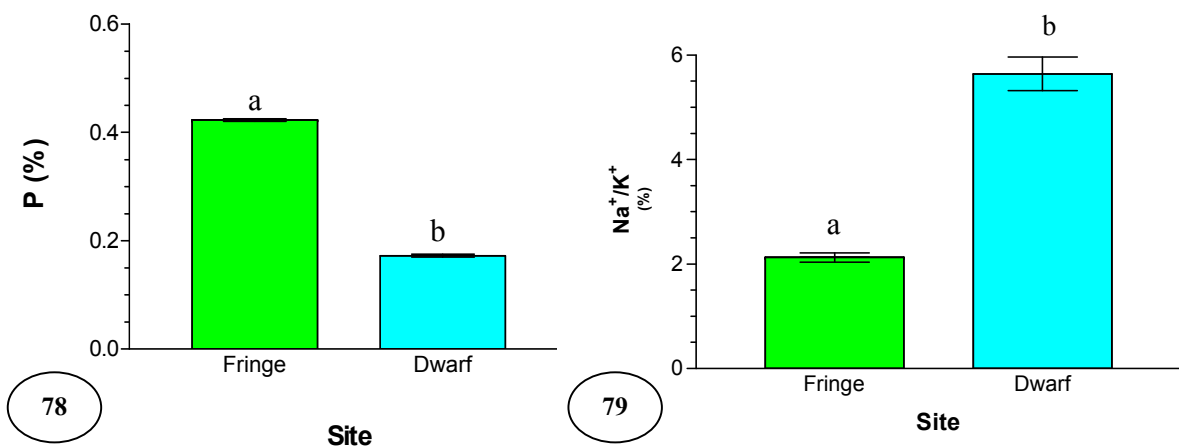
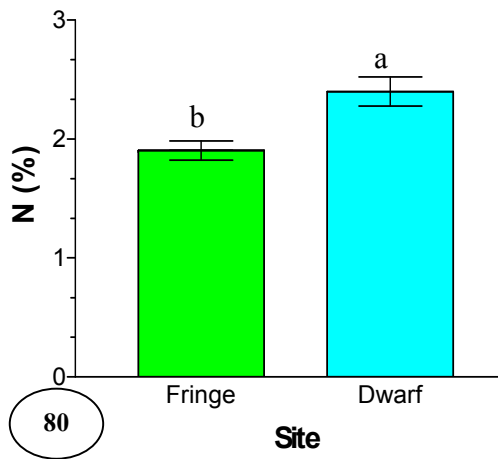
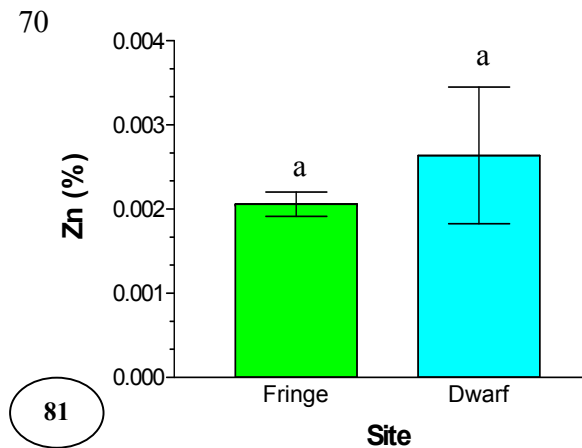


Fig. 78. Phosphorus concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 75.

Fig. 79. Na⁺/K⁺ ratio in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 6). Other details as for Fig. 75.



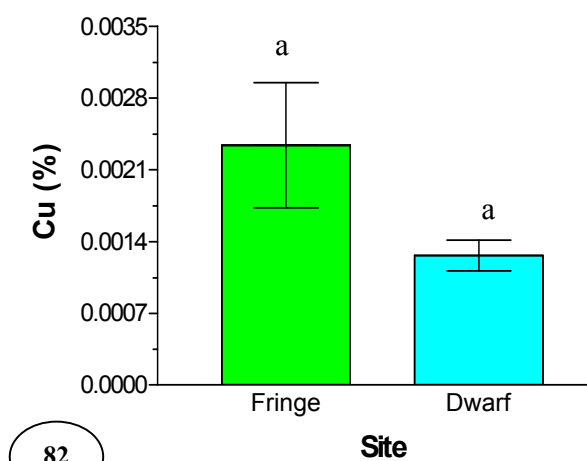
80



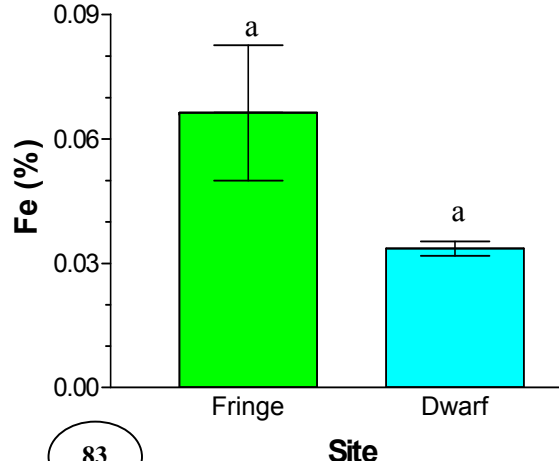
81

Fig. 80. Nitrogen concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 75.

Fig. 81. Zinc concentration in leaves of fringe and dwarf mangroves. Values are means \pm SE (n = 10). Bars with the same letter show no significant difference.



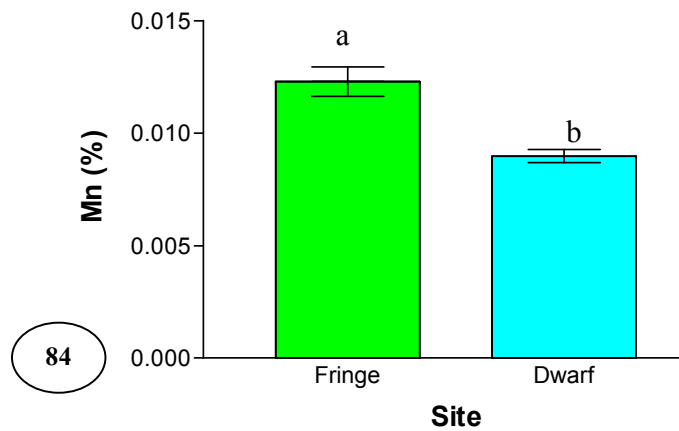
82



83

Fig. 82. Copper concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 81.

Fig. 83. Iron concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 81.



84

Fig. 84. Manganese concentration in leaves of fringe and dwarf mangroves. Other details as for Fig. 75.

CHAPTER 5

DISCUSSION

5.1. MORPHOLOGY AND ANATOMY

Mangroves were found to be a diverse group of tropical trees and shrubs dominating the majority of protected coastal zones and were among the most dynamic systems on earth (Alongi *et al.*, 1992; Tomlinson, 1986; Duke, 1992; Twilley *et al.*, 1992). *Avicennia* is a pantropical mangrove occupying intertidal marine habitats, which are characterised by considerable variations in salinity (Gordon, 1993). Mangroves were found to be proliferated abundantly in estuaries of low salinity levels and were able to tolerate exposure to hypersaline conditions that exceed concentrations of sea water (Naidoo, 2006). However, the consequences of hypersaline environments seemed to present stressful or adverse effects on the growth of mangroves, which appeared to be impaired and stunted (Lugo & Snedaker, 1974; Ewel *et al.*, 1998; Naidoo, 2006). Stunted growth has been also reported as a general salinity-induced effect in plants (Greenway & Munns, 1980; Naidoo, 2006). Dwarf mangroves seemed to grow in areas of limited water availability, high soil salinity at higher elevations, high evapotranspiration, and accumulation of salts (Naidoo, 2006). Dwarf mangrove production has also been attributed to nutrient limitation, especially nitrogen and phosphorus (Feller, 1995; Lovelock *et al.*, 2004; Naidoo, 2006). This is evident in the present study as there was also a decline in the stature of *A. marina* as a result of the hypersaline conditions (60 ‰) along higher elevations in the dwarf zone (Naidoo, 2006). The decline in stature and poor productivity of halophytic plants have resulted due to specific ion toxicity, water and osmotic stress ultrastructural damage and lower carbon assimilation capacity (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1968; Munns & Passioura, 1984; Naidoo, 1986; Hwang & Chen, 1997; Naidoo & von Willert, 1999; Sobrado & Ball, 1999; Munns, 2002; Naidoo, 2006).

In the present study, focus is directed specifically to morpho-anatomical changes in leaves of fringe and dwarf mangroves. Our results confirmed that there were distinct differences in leaf morphology and ultrastructure of fringe and dwarf mangroves. Certain morphological traits such as specific leaf area, leaf and cuticle thickness and salt gland frequency were determined in this study. It has generally been established that the smaller the size of plant organs growing in hypersaline or nutrient limited conditions, the lower the transpiration rates and the resource

demands of the plant (Naidoo, 2006). Naidoo & Mundree (1993) reported salinity-induced reductions in plant height in *Sporobolus virginicus*.

Numerous anatomical differences were evident in leaves of dwarf and fringe mangroves. In comparison to the cuticle in leaves of fringe mangroves, dwarf mangroves developed a significantly thicker cuticle probably to reduce transpiration and heat loading and shield the leaves from damage caused by salt spray (Gorham *et al.*, 1985).

Dwarfism is a feature common to mangroves growing under stressed conditions (Lovelock *et al.*, 2004; Naidoo, 2006). Many studies have indicated that the generation of leaf area is the major factor of plant productivity (Kriedmann & Sands, 1986). There is a reduction in the ratios of leaf area and leaf weight when plants grow under conditions of water stress, thereby minimising transpiration, salt loading in leaves and heat load, but possibly at the cost of productivity (Ball & Passioura, 1995; Fernandez *et al.*, 2002). Additionally, thicker leaves are associated with smaller specific leaf area in drought plants (Fernandez *et al.*, 2002). These findings are in agreement with the reduced specific leaf area, lower relative water content and thicker leaves in dwarf mangroves compared to fringe mangrove leaves (Hiralal *et al.*, 2003). According to Ball & Passioura (1995), a small leaf area in mangroves develops boundary layer conductance so that leaf temperature is close to that of ambient air, without any barrier with light interception.

Scanning electron microscopy (SEM) showed that both adaxial and abaxial leaf surfaces were interspersed at regular intervals with multicellular salt glands. These findings were in agreement with early studies of Fahn & Shimony (1977); Drennan & Berjak (1982); Liphshitz & Waisel (1982); Thomson *et al.* (1988) and Naidoo & Chirkoot (2004). Abundant salt crusts and exudates were found on and bordering salt glands. It is assured these areas might have a significant role in the exit of salt secretions from the leaves. They were further related to the abundance of salt glands in halophytes, *Limonium vulgare* and *Spartina anglica* suggesting high secretion rates. Salt glands appeared protruding from individual crypts and occurred more frequently at different loci on the adaxial leaf surfaces of fringe mangroves compared to those of dwarf mangroves. This evidence is also supported by Rozema *et al.* (1981) and Drennan & Berjak, (1982). Berjak (1977) correlated glandular frequency with leaf age or expansion, where the average number of glands (8 per mm²) on the young *A. marina* leaf invariably exceeded the average number on a mature leaf (0.5 per mm²). This study, therefore provides clear evidence of the differences in

cellular glandular morphology and ultrastructure as well as glandular frequencies between fringe and dwarf mangrove leaves.

It appeared that, on the abaxial leaf surface, non-glandular trichomes probably served to reduce light incident on photosynthetic tissue and to protect protruding salt glands and stomata from exposure to desiccation and evapotranspiration as it has been suggested in other species in the literature (Waisel, 1972; Rozema, 1991; Naidoo *et al.*, 2002; Naidoo & Chirkoot, 2004). The density and overlap of trichomes appear to shield and protect the glands and stomata from salt spray reaching the living leaf tissues. Stomata are commonly found in a protected position among surrounding trichomes and abaxial salt glands. This organisation of leaf structure seemed to accommodate maximum gas exchange while establishing a shallow diffusion gradient for water vapour exiting the leaves. Furthermore, it appeared that stomata have the ability to minimize water loss while only marginally limiting carbon gain in stressed plants (Farquhar & Sharkey, 1982; Raven, 2002; Brodribb & Holbrook, 2003). Reduced stomatal conductance is commonly associated with increased salinity stress (Longstreth & Strain, 1977). This is an indication that salt glands are complex structures adapted for dealing with salt accretion in leaves. According to Flowers & Yeo (1986) and Tomlinson (1986), species with salt glands such as *A. marina* are able to tolerate higher salt concentrations in the xylem than those without glands. Thus, it appeared that, in *A. marina*, salt glands are a remarkable adaptation for regulating the internal ionic concentrations by eliminating excess salts and ions that could reach near toxic levels. It is assumed that the glands probably lower apoplastic salt loads thereby reducing transpirational losses in the leaf tissues (Fitzgerald & Allaway, 1991).

Evidence from SEM studies demonstrated that salt exudates emanated directly from the glands, suggesting that the glands were the specific sites of active salt secretion. Similar observations were reported for other halophytic species (Waisel, 1972; Hansen *et al.*, 1976; Lipschitz & Waisel, 1982). Heavier deposition of crystalline salts was found occluding the adaxial glands of dwarf mangrove leaves, as compared to those of fringe mangroves, which might be attributed to greater concentration of salt in the transpiration stream of dwarf mangrove leaves. This was consistent with findings of Munns (2002) who found that salts moving in the transpiration stream were deposited in leaves as water evaporates, thereby causing the gradual accretion of salt with time. Hill & Hill (1976) reported similar findings as a result of a continual loss of water by evapotranspiration.

The bulbous globules of salt secretions produced by the glands suggested that salts were secreted in small quantities at the base of the crystal formation, which appeared to amass on and around the gland. As suggested for *A. marina* (Roshchina & Roshchina, 1993), it is possible that the nature of crystal formation, which might be either a cluster of small crystals, large cube-shaped crystals or large lamellar crystals, which was dependent on minor variations in conditions in the immediate environment of the gland at the time of crystallization.

Salt glands of dwarf mangrove leaves appeared to be deeply sunken within crypts, probably due to lower water availability, higher evapotranspiration and desiccation in the dwarf zone. It is suspected that, as salts move along the transpiration stream in leaves and evaporation of water takes place, salts gradually build up in the leaves with time, as it was also suggested by Munns (2002). It appeared that, to counteract specific ion toxicity, leaves of dwarf plants eliminated salt to the surface via the glands. Thus, when the mechanism controlling glandular secretion in the mature leaves of dwarf mangroves cannot cope with eliminating salts to the surface, and the ionic concentration within the leaf tissues reach toxic levels, salt glands plasmolysed, then probably degenerated and finally senesced (Munns, 2002). The dwarf zone received infrequent tidal coverage (only at high spring tide), and plants were water stressed. As a result, dwarf mangrove leaves managed to conserve water and subsequently prevented desiccation by developing thicker leaves and a thicker cuticle (Hiralal *et al.*, 2003). However, the fringe mangroves received daily inundations of seawater thereby maintaining homeostatic ionic and water balance. Studies on fringe and dwarf *A. marina* showed that the soil water potentials decreased with increasing distance from the water's edge, being -3.2 MPa in the fringe site and -7.67 MPa in the dwarf site (Naidoo & Chirkoot, 2004). Similar findings were reported by Naidoo (2006) who demonstrated that in the fringe site, soil water potential was -3.07 MPa and xylem water potential was -4.5 MPa, thereby suggesting a positive water balance. In the dwarf site, there was a decrease in soil water potential (-7.44 MPa) and xylem water potential of -6.5 MPa, resulting in plant water deficits.

Additionally, trees in the fringe zone appeared to flourish along the waters edge possibly showing that plant organs were functioning optimally in transport of ions and secretion of salts, without the threat of ionic imbalances, degeneration and morpho-anatomical alterations.

5.2. ULTRASTRUCTURAL STUDIES

The present study has shown that there were differences in ultrastructural morphology in leaf cells of dwarf and fringe mangroves.

The swelling and disintegration of organelles such as chloroplasts, mitochondria, vacuolar tonoplast and nuclei are common features displayed by plants growing in highly saline soils and was suggested to be a response to the changing internal environment of plant tissues (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1968; Poljakoff-Mayber, 1975; Werker *et al.*, 1983). Ultrastructural work in this study has supported these findings. The discovery of increased formation of vesicles and myelin-like structures in the vacuoles of plants growing in saline soils were probably pinocytic invaginations of the tonoplast, which allowed plants to mobilize ions from the cytoplasm into the vacuoles (Willert & Kramer, 1972; Kurkova & Balnokin, 1994). Details of multivesicular structures outlined above were a common feature in dwarf mangrove leaves.

The cuticle and epidermis

The cuticle and epidermal cells of leaves of dwarf mangroves compared with those of fringe mangrove leaves were characterised by a thicker cuticle and discontinuous, irregular-shaped epidermal cells, probably as a means to conserve water, and to prevent evapotranspiration and desiccation. Other studies showed that plants like mangroves, salt marsh plants and sea grasses respond similarly when subjected to salinity stress. Flowers & Yeo (1986), for example, reported a 1.6-fold increase in cuticle thickness in the salt marsh plant, *Suaeda maritima* under saline conditions. Jagels & Barnabas (1989) and Iyer & Barnabas (1993) observed fine structural damage in leaf epidermal cells of sea grasses, *Ruppia maritima* and *Zostera capensis* at salinities ranging from 40 – 60 ‰.

Hypodermis

In *A. marina*, the hypodermis formed part of the mesophyll and is composed of layers of colourless water storage cells below the adaxial epidermis (Tomlinson, 1986). The walls of the hypodermal cells were thicker and wavy in dwarf as compared to those in fringe mangrove leaves. Plasmolysis was evident in dwarf mangrove leaves as a distinct withdrawal of the plasma

membrane and cytoplasm from the hypodermal cell wall forming an extracytoplasmic space, possibly due to a response to increased ion sequestration and osmotic stress. It was noted that plasmolysis was absent in fringe mangrove leaves; thereby suggesting that plasmolysis was not a fixation artifact.

Mesophyll (Palisade and Spongy)

In *A. marina* leaves, the mesophyll comprises well-developed, thick layers of palisade cells arranged parallel to each other below which were large rounded spongy cells with intercellular airspaces. Structural alterations in chloroplasts, mitochondria, nuclei, vacuoles were visible in mesophyll tissue of leaves from dwarf plants.

Differences between palisade mesophyll cell ultrastructure in fringe and dwarf mangrove leaves.

Marked changes in palisade mesophyll from leaves of dwarf plants included morphological alteration in chloroplasts, mitochondria, cell wall-plasma membrane displacement and the presence of multivesicular structures. In dwarf mangrove leaves, starch grains within the chloroplasts were reduced in size possibly due to lower rates of photosynthesis and water stress. However, plastoglobuli (lipid globules) appeared increased in size and number in dwarf leaf chloroplasts. A similar observation was made in many studies on the effects of high NaCl on plants (Kutik *et al.*, 1999; Migge *et al.*, 1999). The increase of plastoglobuli (number or size) was found to be one of the most characteristic phenomena accompanying chloroplast senescence (Kutik, 1998, Hudak, 1997). In agreement with Nir *et al.* (1970), plastoglobuli were found associated with salt-stressed chloroplasts and disruption of membranous systems. The appearance of large plastoglobuli might be due to an accumulation of lipid as a consequence of partial thylakoid breakdown (Migge *et al.*, 1999). The chloroplasts of dwarf mangrove leaves were distorted with swollen thylakoids and stroma lamellae, mitochondria appeared to be highly translucent, with dilated cristae and nuclei appeared distorted. However, in fringe mangrove leaves, palisade leaf cells showed elliptical chloroplasts with an intact structural integrity. Large starch grains and smaller plastoglobuli were observed in chloroplasts of fringe mangrove leaves suggesting higher photosynthetic activities in these leaves.

Differences between spongy mesophyll cell ultrastructure in fringe and dwarf mangrove leaves.

Spongy mesophyll cells in leaves of dwarf mangroves showed evidence of ultrastructural changes in organelles. The plasma membrane appeared to be detached from the cell wall as a result of plasmolysis and the nuclei appeared fragmented. However, spongy mesophyll cells from fringe mangrove leaves maintained an integrated structure. The nucleus remained oval with an intact nuclear envelope, chloroplasts appeared to be elliptical in shape with large starch grains and well developed granal stacks interconnected with integranal lamellae and the two membranes of the chloroplast envelope were intact, indicating that fringe mangroves were not negatively affected by environmental stresses. Swelling of thylakoids, distortion of chloroplasts and the presence of smaller and fewer starch grains were observed possibly due to stressed conditions of high soil salinity and low water availability. High salt concentrations in the leaves of dwarf plants probably caused the formation of vesicles on the chloroplast envelope, as well as unstacking of grana and disintegration of chloroplast envelope, which caused the stromal contents to mix with the surrounding cytoplasm. Accumulation of plastoglobuli in the chloroplast stroma might be due to premature ageing or senescence of dwarf leaves that could not tolerate the build-up of salts within the leaves. Severe salt stress altering the ultrastructure of halophytic plant organelles has been reported by several investigators (Pareek *et al.*, 1997; Rahman *et al.*, 2000).

Chloroplasts

As a coping mechanism, plants growing under saline conditions appeared to change their ultrastructure and morphology. Alternatively, saline conditions have shown to induce ultrastructural and morphological alterations to the plant (Harvey & Thorpe, 1986). Furthermore, numerous studies on salinity affecting plant structure have been well documented (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1968; Kelly *et al.*, 1982; Hernandez *et al.*, 1995; Rahman *et al.*, 2002). Since chloroplasts were shown to play a vital role in plant tolerance to salt stress according to Schroppel-Meier & Kaiser (1988a,b), in the present study, there were distinct differences in chloroplast ultrastructure between fringe and dwarf mangrove leaves. In fringe mangrove leaves, chloroplasts appeared to be elliptical with large starch grains occupying most of the chloroplast volume. The granal stacking contained more than 11-20 thylakoid layers, suggesting that *A. marina* in the fringe zone was healthy and functioning optimally. However, in

dwarf mangrove leaves, there were many structural changes in chloroplasts as evidenced by swelling (i.e. separation of the thylakoid membranes). Such swelling of granal and lamellar compartments of chloroplasts were observed in bundle-sheath chloroplasts, mesophyll, epidermal and hair cells of salt marsh *Atriplex halimus* (Kelly *et al.*, 1982). In addition, salinity caused ultrastructural changes in leaves of rice seedlings, inducing swelling of thylakoids, disintegration of granal stacking and integranal lamellae and mitochondrial damage (deficiency of cristae, swelling and vacuolation) (Rahman *et al.*, 2002). Blumenthal-Goldschmidt & Poljakoff-Mayber (1968) also reported a similar swelling of chloroplast lamellae and mitochondrial cristae in *A. halimus* growing under highly saline conditions, and in extreme cases, the swelling was so pronounced that the granal structure became unrecognisable. Some thylakoid undulation, disruption of the integranal lamellae and chloroplast envelope could have adversely affected photosynthesis in dwarf mangroves. Amongst numerous studies on the effects of salts on chloroplasts were those by Ball & Anderson (1986) who reported that the accumulation of sodium chloride in the chloroplasts of salt-tolerant *A. marina* and salt sensitive pea, *Pisum sativum* resulted in rapid damage to photosystem II, particularly in light. Muller & Santarius (1978) reported that high concentrations of sodium chloride have damaging or toxic effects on thylakoids, inhibiting photosynthesis. Migge *et al.* (1999) also correlated changes in the ultrastructure of chloroplasts with a decline in the rate of net photosynthetic CO₂ uptake. Mitsuya *et al.* (2000) and Yamane *et al.* (2003) observed ultrastructural changes in chloroplasts of salt-treated rice plants, suggesting that the ionic effect of sodium chloride induced swelling of thylakoids and the osmotic effects caused a destruction of the chloroplast envelope. In this study, trees in the fringe zone effectively secreted salts, and chloroplasts and other plant organelles appeared to be undamaged. However, due to reduce water availability and high salt concentration in the dwarf zone, dwarf mangroves found it more costly to cope with the greater salt load and the photosynthetic machinery within the chloroplasts become damaged, reducing or inhibiting photosynthesis. Krupa & Baszynski, (1989) reported that the disorganization of photosystem II structure occurred because saline conditions lower the K⁺ ion concentration, which was crucial for water status. Stomatal conductance, rubisco activity and photosynthetic rate were reduced under salt stress in mangroves (Walker *et al.*, 1981; Roebel *et al.*, 1982; Ball & Farquhar, 1984; Yeo *et al.*, 1985, Kumar & Gupta, 1986; Bongi & Loreto, 1989; Naidoo & Chirkoot, 2004). Additionally, the accumulation of salts within leaf tissue might reach near-toxic levels where salts could not be effectively eliminated or sequestered thereby affecting the structural components of chloroplasts, mitochondria, cell wall and plasma membrane, vacuoles and nuclei. Amoeboid deformations of chloroplasts in dwarf mangrove leaves were evident in this investigation. Similar

findings have been reported in the salt marsh *A. halimus* (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1968) and epidermal leaf cells of the sea grass *Zostera capensis* (Iyer & Barnabas, 1993).

In the present investigation, a characteristic feature of chloroplasts in mature dwarf mangrove leaves was the accumulation of plastoglobuli, which was probably a response to salt stress, and leaf ageing or senescence (Kutik *et al.*, 1999). Extensive lipid accumulation occurred in some of the chloroplasts of salt-treated plants of *A. halimus* (Kelly *et al.*, 1982). Thomson (1975) reported that plastoglobuli occurred in many types of chloroplasts in a wide range of plants and suggested that their occurrence was not due to a response to stress but to the “ageing” of chloroplasts. Hernandez *et al.* (1995) reported that in chloroplasts of pea plants, plastoglobuli increased in number and size as a result of salt stress. Kutik *et al.* (1999) suggested that growth of plastoglobuli was a response to chloroplast maturity and senescence.

Mitochondria

Conspicuous structural damage to mitochondria was noted in dwarf mangrove leaves. The mitochondrial envelope appeared to be fragmented; cristae were vacuolated and swollen. These results were consistent with those reported for *Agrostis stolonifera* (Smith *et al.*, 1982), *Z. capensis* (Iyer & Barnabas, 1993) and rice seedlings (Yamane *et al.*, 2003) in response to salinity. Smith *et al.* (1982) and Rahman *et al.* (2002) suggested that damage to mitochondria could be a direct effect of ions within the tissue or water stress imposed by salt. The decrease in electron density within the mitochondrial matrix appeared to reflect the disintegration of the mitochondria and therefore a decrease in mitochondrial activity. Kelly *et al.* (1982) reported that in salt-stressed plants of *A. halimus*, mitochondria lacked cristae due to osmotic disruption and swelling. Fringe mangrove leaves exhibited mitochondria that were well distributed throughout the mesophyll cells appearing round to tubular with a distinct envelope and well developed cristae in contrast to those from dwarf plants.

Cell wall and plasma membrane

Withdrawal of the plasma membrane and cytoplasm from the cell wall was a structural response to highly saline conditions. In the present study, the cell wall of fringe mangrove leaves remained intact and the plasma membrane remained closely appressed to the cell wall. However, in dwarf

leaves the plasma membrane was withdrawn from the cell wall at several points indicating plasmolysis which might be a consequence of excess ion build up within the cell wall and or osmotic stress (Pareek *et al.*, 1997; Mitsuya *et al.*, 2002). Withdrawal of the plasma membrane from the cell wall as a result of the accumulation of toxic salts was reported in several studies (Nir *et al.*, 1969; Kalaji & Pietkiewicz, 1993; Mitsuya *et al.*, 2000; Rahman *et al.*, 2000). Salinity was also shown to cause cell wall thickening (Iraki *et al.*, 1989; Singh *et al.*, 1987). Plasmolysis and cytoplasm degradation appeared to be an indicator of cellular damage induced by salt (Pareek *et al.*, 1997; Mitsuya *et al.*, 2002). Formation of vesicles was observed in the extracytoplasmic space created by the withdrawal of the plasma membrane from the cell wall in dwarf mangrove leaves. These vesicles appeared to resemble plasmalemmasomes, which were similarly reported in leaf epidermal cells of *Z. capensis* (Barnabas *et al.*, 1977).

Vacuoles and associated multivesicular structures

Multivesicular structures featured commonly in dwarf mangrove leaves and were located in the vacuoles, chloroplasts, mitochondria and along cell walls and plasma membranes compared to those from fringe mangrove leaves. Plants growing at high salinities showed an increase in the presence of vesicles and myelin-like structures in their vacuoles (Mitsuya *et al.*, 2000). The presence of myelinated figures and plasma strands in vacuoles of mesophyll cells in *A. halimus* was similar to those reported in the present study. These structures were believed to be pinocytic invaginations of the tonoplast, allowing plants to translocate ions from the cytoplasm into vacuoles (Willert & Kramer, 1972; Kurkova & Balnokin, 1994). Vacuolation has shown to be an adaptive response to compartmentalise or sequester sodium ions away from the cytoplasm (Braun *et al.*, 1986; Match *et al.*, 1987; O' Leary, 1995; Blumwald *et al.*, 2000). However, enzyme activity is inhibited when ions built up in the cytoplasm and cell wall, causing the cell to dehydrate and lose turgor (Munns & Passioura, 1984; Flowers & Yeo, 1986). The rate at which ions increased in the cytoplasm would be greater than the rate of increase in the vacuole because the cytoplasmic space was much smaller than that of the vacuole (Munns, 2002). Therefore the formation of vesicles and multivesicular structures within the vacuoles and surrounding cytoplasm might be a response to an increase of salt concentration. Thus, ion compartmentalisation within these structures might assist in osmoregulation and deposition of ions that were toxic to the cell (Yeo *et al.*, 1990; Maathius *et al.*, 1992). Fringe mangroves exhibited fewer multivesicular structures within the leaf cells compared to dwarf plants and were

probably capable of efficient osmoregulation and maintenance of an ionic balance between the cytoplasm and vacuoles.

Salt glands

Ultrastructural features of salt glands suggested that these structures are elaborately adapted for coping with salinity and other environmental stresses such as coal dust and heavy metal accumulation on the leaves. These salt glands seemed to be structurally adapted to play a vital role in salt secretion and regulation of ion concentration in leaves, thereby preventing the toxic buildup of ions according to the documented literature (Naidoo & Naidoo, 2001). It has been further shown that the salt glands of *A. marina* are multicellular, consisting of specialised cells that seemed to eliminate excess salt to the leaf surface (Naidoo *et al.*, 2002). This evidence is also supported by Fitzgerald & Allaway (1991) who have suggested that the pathway of ion movement was through both apoplastic and symplastic routes in the salt glands.

Ultrastructurally, the basal collecting cells were found to be highly vacuolated and occurred at the epidermal level. It was noticed that cuticle enclosed the single stalk cell wall whilst a relatively thin cuticle encloses the eight, sometimes twelve terminal secretory cells, which are separated apically from the non-cutinised wall in order to leave a shallow cavity called the cuticular chamber. The lack of chloroplasts and the presence of numerous mitochondria in gland cells, suggested intense glandular activity. This structural evidence is consistent with findings of Shimony *et al.* (1973), Fahn & Shimony (1977) and Tomlinson (1986) who have conducted extensive studies on salt gland ultrastructure in *A. marina* leaves.

This study has, therefore, managed to elucidate some of the ultrastructural adaptation as fringe mangroves are exposed to daily tides, salty winds and salt spray. Thus, the roots are able to exclude salt by ultra-filtration, preventing the transpiration stream from becoming more concentrated than seawater. The ions that entered the transpiration stream were actively secreted by salt glands onto leaf surfaces. The stalk and secretory cells of fringe mangroves possessed a dense cytoplasm rich in ribosomes, large nuclei and many mitochondria, indicating high metabolic activity. This high metabolic activity has been indirectly supported by Scholander (1968) and Shimony *et al.* (1973) who described the symplastic movement of salts from the trichomes across a decreasing ionic gradient into the secretory cells, through the expanded cuticular chamber and then via the cuticular pores to the leaf surface where salts accumulated.

The leaf cuticle and cutinised wall of the basal cell prevents apoplastic backflow (Tomlinson, 1986).

The cuticular cavity or chamber was in salt glands of both fringe and dwarf mangroves. It is assumed that it probably served as a temporary collecting compartment where secreted salts accumulated prior to exit from the leaf, as it was also suggested by Campbell & Thomson (1976). TEM data of salt glands from fringe and dwarf leaf mangroves showed the distension of the cuticle which was probably due to the increase in hydrostatic pressure created within the gland, which is assumed to play a role in eliminating excess salts through the terminal cell wall, and possibly the cuticular pores. This possibility has been confirmed by Shimony *et al.* (1973).

Furthermore, Shimony *et al.* (1973) observed that vesicles found in salt glands of *A. marina* have a secretory function. In both fringe and dwarf leaf mangroves, vesicles of varying sizes common in salt glands were probably involved in secreting wall material and wall synthesis as it was also recommended in the literature (Rachmilevitz & Fahn, 1973; Shimony *et al.*, 1973). Smaller vesicles were also noted between the retracted cytoplasm and cell wall of the secretory cells. These vesicles found in gland cells were involved in salt secretion (Thomson & Liu, 1967; Shimony & Fahn, 1968; Osmond *et al.*, 1969; Shimony *et al.*, 1973). In agreement with Shimony *et al.* (1973), this study showed the presence of secretory cells which contained elongated vacuole-like structures. This might be an indication that these vacuole-like structures had a most active part to play in salt secretion, as fibrillar, band-like structures became distended when the concentration of salt in the gland was high. It was suggested that the “band” membranes originated from the Golgi apparatus (Shimony *et al.*, 1973).

The cell wall and cytoplasm of the secretory cells appeared to be retracted in dwarf mangrove leaves, suggesting plasmolysis. Numerous larger vacuoles occupied the stalk and secretory cells, indicating that ions are sequestered into the vacuoles, which then discharged their salty contents to the exterior (Tomlinson & Liu, 1967, Shimony & Fahn, 1968). The electron dense material in the vacuoles could be pectinaceous in nature and served as carriers for salt translocation (Tomlinson & Liu, 1967, Shimony & Fahn, 1968). The high vesicular volume of all glandular cells of dwarf mangrove leaves suggested that vesicles play an important role in salt secretion.

Trichomes

Peltate trichomes were the non-glandular hair-like structures that form a dense overlapping indumentum that was confined to the abaxial surface of *A. marina* leaves. It is assumed that the hairs (trichomes) were part of the agents, which assisted the mechanism that fortified the cuticle, protecting the leaf from salt accumulation and reducing water loss. Additionally, the extension of the cuticle over the trichomes and glands appeared to serve as an apoplastic block, preventing salts from entering the leaf through the stomata on the abaxial epidermis from the outside (Fitzgerald & Allaway, 1991).

Stomata

Stomata were found to be housed between the abaxial salt glands and overlapping trichomes and shielded from desiccation. It appeared that their arrangement facilitated an optimal gas exchange while creating a shallow diffusion gradient for water vapour exiting the leaves. High vacuolar content in the guard cells of stomata was presumably a response to the reduced water and carbon fluxes and an increase in ionic concentrations thereby lowering the photosynthetic performance and stomatal conductance in leaves. To prevent ion toxicity within the leaf tissue and to limit evapotranspiration, it appeared that the stomata have developed ways of coping with water deficits, lowered CO₂ exchange and ionic imbalances in the dwarf site. It is suspected that cell wall thickening and high vacuolar volume might be ultrastructural modifications which dealt with water loss and desiccation reduction.

5.3. PHYSIOLOGY

The physiological responses of mangroves and other halophytic plants have been extensively studied (Waisel *et al.*, 1986; Naidoo & Naidoo, 2001; Cram *et al.*, 2002; Mühling & Läubli, 2002; Ye *et al.*, 2005; Naidoo, 2006). Physiological studies were undertaken in this investigation to compare differences in osmotic and ionic relations as well as whole plant responses in fringe and dwarf mangrove leaves.

5.3.1. % Relative leaf water content (% RWC)

Our investigations showed that the % relative leaf water content were lower by 7.8 % in dwarf mangroves, which was in agreement with observations made by Naidoo (2006) for dwarf mangroves. The dwarf site received tidal inundations only during high spring tides and the soils were salt-encrusted and hardened due to the lack of available water and higher evapotranspiration. Leaves of dwarf plants had an insufficient supply of water in the transpiration stream of roots, stems and leaves and consequently, a lower % RWC compared to fringe mangroves leaves. A significant reduction in % relative leaf water content and a decrease in leaf chlorophyll content in the dwarf site is considered to result in impaired RUBP regeneration and ATP synthesis in dwarf mangroves, as it has been also documented in the literature (Lawlor & Cornic, 2002; Naidoo, 2006).

5.3.2. Specific leaf area (SLA)

Fringe mangrove leaves showed a greater specific leaf area by 17 % than those of dwarf mangroves probably due to higher water availability in the fringe site. Dwarf mangrove leaves had a lower SLA probably to lower their transpiration rates and salt loading. Our findings were in agreement with Fernandez *et al.* (2002), who found that water-stressed plants were characterised by a tendency to have a smaller proportion of their biomass as leaves (i.e. reduced leaf weight ratio), thicker leaves and smaller specific leaf area which led to reduced plant growth.

5.3.3. Leaf thickness and cuticle thickness

Leaf thickness is determined by the irradiance at leaf development and shows little change after maturity (Oguchi, 2003). Dwarf mangroves had 27.6 % thicker leaves than fringe mangroves.

The thickening of dwarf mangrove leaves is associated with a xerophytic feature which is adapted by dwarf mangroves in order to store or conserve water due to the reduced water availability in the dwarf site; and reduced further loss by evapotranspiration (Hiralal *et al.*, 2003). Leaf thickness in *Phaseolus vulgaris*, *Gossypum hirsutum* and *Atriplex patula* (Valenti *et al.*, 1992) was also reported to increase with increases in salinity, so as to conserve water.

5.3.4. Chlorophyll content

Fringe mangrove leaves displayed higher chlorophyll content than those of dwarf mangroves. Our results were consistent with those of Naidoo (2006) who reported that leaf chlorophyll content was higher in fringe mangroves and significantly reduced by 24 % in dwarf mangroves. In addition, % relative leaf water content and water availability was reduced which possibly indicated that RUBP regeneration and ATP synthesis was impaired in dwarf mangroves, as it has been also documented in the literature (Lawlor & Cornic, 2002). Ball *et al.* (1987) reported that leaves from high salinity/low K⁺ treatment were characterised by drastic losses in chlorophyll concentration and photosynthetic capacity as well as photochemical dysfunction. Additionally, the decline in chlorophyll content and photosynthetic activity might have led to leaf senescence as it was also described in literature (van Doorn & Woltering, 2004; Wingler *et al.*, 2006).

5.3.5. Salt gland frequency

In the present study, mature fringe mangrove leaves showed a higher glandular frequency than mature dwarf mangrove leaves. It is therefore, suggested that leaves of fringe mangroves were actively involved in secreting salts at a higher rate and efficiency than dwarf mangrove leaves. It was also noted that the frequency of glands increased from the leaf apex to basal regions in both fringe and dwarf mangrove leaves. It might be possible that leaves have structurally positioned their salt glands more densely at basal loci rather than towards the apex in order to eliminate salts from the transpiration stream more rapidly via the nearest possible route to the surface. Rozema *et al.* (1977) reported an increased number of glands in leaves of *Glaux maritima* grown under high salinity.

5.3.6. Osmotic relations:

Glycinebetaine and proline

In the present study, the concentration of glycinebetaine was 40 % higher in leaves of dwarf than in fringe plants. The same effect has been demonstrated in several reports on salt and water stressed plants (McCue & Hanson, 1990; Rhodes & Hanson, 1993; Kishitani *et al.* 1994; Popp & Albert, 1995; Sakamoto & Murata, 2002). Furthermore, glycinebetaine is known to occur extensively in different *Avicennia* species and it is known to act as a compatible osmoticum to overcome the toxic effects of Na⁺ and Cl⁻ (Popp & Polania, 1989). Popp & Albert (1995) reported that *A. marina* accumulates large amounts of glycinebetaine to maintain osmotic balance. Marcum & Murdock (1992) cited in Khan *et al.* (1998) showed that glycinebetaine accumulated in shoot tissues of *Sporobolus virginicus* with increasing salinity and proposed that glycinebetaine might act as a compatible solute. Cavalieri (1983) determined that total glycinebetaine content was approximately ten times higher than proline content in the salt marsh grass *Spartina alterniflora*.

The findings of this study and the documented evidence in the literature which is discussed in the above paragraph with regard to glycinebetaine showed that plants have developed protective mechanisms that allowed them to acclimatize to unfavourable environments, such as water deficits and hypersalinity, for continued growth and survival. Proline levels in *A. marina* and other mangroves were low and it appeared that proline levels were, therefore, not important in osmotic balance as in glycinebetaine. Concentrations of proline were 27 % lower in dwarf than in fringe mangrove leaves, suggesting that the concentration of proline was not sufficient to play a significant role in the osmotic balance of the cell (Aziz & Khan, 2000). It was found that one such mechanism that is ubiquitous in plants, was the accumulation of compatible solutes (Bohnert *et al.* 1995). Glycinebetaine served as a compatible solute in osmotic adjustment of the cytoplasmic compartments where it might accumulate while ions were sequestered in the vacuole and was able to also 'protect' protein and membrane functions from high concentrations of Na⁺ and Cl⁻ (Robinson and Jones, 1986; Match *et al.* 1987; Rhodes & Hanson, 1993; Bohert *et al.* 1995; Hanson *et al.* 1995). According to Rahman *et al.*, (2002) salinity induced ultrastructural damages in leaves of rice seedlings such as swelling of thylakoids, disintegration of grana stacking and integranal lamellae and destruction of mitochondria were largely prevented by pretreatment with glycinebetaine resulting in greening of the plants. In addition, Munns (2005) was of the view that a high production of glycinebetaine and other osmoprotectants within

stressed plants would carry an energy cost if their accumulation were to maintain cell turgor, which might be consequential to plant growth. It is, therefore, considered that, even in the presence of high internal salt concentrations, wilting and toxicity would be avoided and survival rate of plants improved with glycinebetaine production. All these documented reports were found to be consistent with the findings of this study.

5.3.7. Leaf ion concentrations

In this study, the predominant ion detected in mature leaves was Na^+ , which was 19 % higher in leaves of dwarf than fringe mangroves. When plants are exposed to NaCl , ions seemed to reduce the apoplastic water potential and accumulate excessively in the cytosol (Binzel *et al.*, 1988). This process has caused a disrupting of compartmentation of ions in the vacuole and led to ion toxicity and membrane damage (Yeo, 1998; Marcar & Termaat, 1990; Kalaji & Pietkiewicz, 1993). This evidence has been demonstrated by the ultrastructural damage in cellular components of dwarf mangrove leaves in this study. Kurth *et al.* (1986) also reported that Na^+ ions had deleterious effects on the structural and functional integrity of membranes. It has been demonstrated that the functioning of the Na^+/H^+ antiport present in the tonoplast to allow compartmentation of ions in the vacuole was likely to be disrupted by high concentrations of Na^+ in the cytosol, thereby decreasing halo-tolerance (Barkla & Pantoja, 1996; Yeo, 1998). Niu *et al.* (1995) concluded that a high Na^+ and Cl^- level in the apoplast was able to alter aqueous and ionic thermodynamic equilibria, resulting in hyperosmotic stress, ionic imbalance and toxicity. Fringe mangroves adapted well to the saline environment by actively compartmentalizing Na^+ and Cl^- ions from cytosol to vacuole. Cl^- movement from the cytosol to the vacuole might be achieved through channels across the tonoplast (Niu *et al.*, 1995). It was suggested that when plants took up salts, plant cell injury resulted because of the toxic effects of Cl^- ions (Brawley & Mathes, 1990). High concentrations of Na^+ in mature leaves has shown to accumulate over long periods leading to specific ion toxicity and impaired nutrient acquisition in salt marsh species and mangroves (Greenway & Munns, 1980; Popp *et al.*, 1984; Flowers *et al.*, 1977; Mühling & Läuchli, 2002). In this respect, halophytic plant growth was inhibited when there was a high proportion of Na^+ ion uptake in relation to other essential inorganic ions like K^+ , Ca^{2+} , Mg^{2+} , P, NO_3^- , Mn^{2+} (Yeo, 1998; Zhu, 2002; Gulzar *et al.*, 2005, Koyro, 2006).

In the present study, fringe mangrove leaves contained significantly higher concentrations of K^+ and lower Na^+ compared to those in dwarf mangrove leaves. The Na^+/K^+ ratio was 62 % greater

in dwarf mangrove leaves than those of fringe mangroves. It is then suggested that high concentrations of Na^+ in the dwarf mangrove leaves impaired uptake of K^+ probably due to physio-chemical similarities between these two elements (Marschner, 1995; Läuchli, 1999b). Under salt stress, cytoplasmic K^+/Na^+ balance had to be maintained at the plasma membrane and tonoplast. Na^+ is sequestered in the vacuole whilst K^+ is loaded into the cytoplasm. It has been shown in our results of dwarf mangrove leaves and in literature that Na^+ outcompeted K^+ at transport sites for K^+ entry into the symplast, which ultimately led to K^+ deficiency (Marschner, 1995; Yeo, 1998). Moreover, it has been demonstrated that cytoplasmic Na^+ competes for K^+ binding sites and could inhibit crucial metabolic processes that depend on K^+ (Marschner, 1995; Gassman *et al.*, 1996; Yeo, 1998; Amtmann & Sanders, 1999).

It should be remembered that potassium is a nutrient that is required in large concentrations for photosynthesis and other metabolic processes (Huber, 1985). The concentration of K^+ in leaves has shown to decrease with an increase in salinity because high concentrations of Na^+ can interfere with K^+ uptake by roots and chloroplasts (Munns *et al.*, 1983; Robinson & Jones, 1986). Ball *et al.* (1987) suggested that reduction in the photosynthetic activity in leaves of *A. marina* under high salinity conditions was due to salinity-induced K^+ deficiency rather than due to the accumulation of NaCl to toxic levels in the leaves. A similar pattern of reduced K^+ concentrations in dwarf mangrove leaves (49 % reduction) was shown in our findings. According to Naidoo (1994), growth of salt-stressed plants is adversely affected with a deficiency in K^+ , suggesting that protein synthesis was affected.

In mangrove forests, hypersalinity was found to be commonly associated with a small forest stature (Cintron *et al.*, 1978; Feller, 1995). The present investigation showed a higher nitrogen concentration (14 %) in leaves from the dwarf site compared to those of the fringe site, which was contrary to findings of Parida & Das (2004) who showed that high salinity decreased the total nitrogen in leaves of the mangrove, *Bruguiera parviflora*, which might affect protein synthesis, and therefore growth and development. In addition, it has been demonstrated that low nitrogen and phosphorus concentrations limited growth of trees (Feller, 1995; Koch, 1997; Feller *et al.*, 1999; 2002; McKee *et al.*, 2002; Lovelock *et al.*, 2004, Naidoo, 2006). High concentrations of nitrogen in dwarf mangrove leaves were due to differences in the contribution of tides and upland influence (Naidoo, 2006). Other studies, however, indicated that fluctuations of nitrogen in mangroves were low and unpredictable with little effect of season and location (Alongi *et al.*, 1992).

Phosphorous concentrations were significantly lower by 50 % in the leaves of dwarf mangroves. Phosphorus limitation in the dwarf site was due to infrequent tidal influence as well as low plant productivity (Naidoo, 2006). The low phosphorus content in the dwarf site has limited nutrient acquisition and microbial processes such as litter decay rates and nutrient retention in rhizosphere (Amador & Jones, 1993). Dwarfing of *Rhizophora mangle* in Panama and Belize (Feller, 1995; McKee *et al.*, 2002; Lovelock *et al.*, 2004) was suggested to be due to nitrogen and phosphorus deficiencies.

It has been demonstrated that Ca^{2+} also improves K^+ uptake under saline conditions, increasing K^+/Na^+ ratio in the tissue, as a salt tolerance mechanism (Cramer *et al.*, 1990; Zhong & Läuchli, 1994). Calcium is important in maintaining membrane integrity when plants are under salt stress (Lynch *et al.*, 1987). However, concentrations of Ca^{2+} were significantly lower in the leaves of dwarf mangroves by 38 %, which is in agreement with other halophytes (Gulzar *et al.*, 2003; Koyro, 2006). Magnesium concentration was also significantly reduced in dwarf mangrove leaves by 35 %. The Ca^{2+} and Mg^{2+} contents were reduced in shoots of *Atriplex griffithii* plants grown at high salinity (Naidoo & Rughunanan, 1990; Ayala & O' Leary, 1995).

It appeared that Na^+ significantly reduced or interfered with uptake of essential elements like, Ca^{2+} , Mg^{2+} , K^+ P and Mn^{2+} thereby inhibiting of plant growth (Popp *et al.*, 1985; Yeo, 1998). Lack of differences in the concentration of microelements, zinc, copper, and iron between leaves of fringe and dwarf mangroves was assumed to attribute to using mature leaves for the analyses. Manganese was 27 % lower in dwarf than fringe mangrove leaves.

CONCLUSION

The sustainability and productivity of *A. marina* in the fringe and dwarf zones appeared to depend largely on tidal inundation and fluctuating salinity. Although *A. marina* was well adapted to survive in the fringe site which received frequent tidal coverage, these plants faced the problem of low water availability and hypersaline conditions at higher elevations which led to poor productivity and stunted growth in the dwarf site.

The investigations conducted clearly confirmed our conclusions. We accept the hypothesis that there are distinct morphological differences between fringe and dwarf mangrove leaves. It has been shown that dwarf mangrove leaves have a lower SLA, thicker leaves and cuticles. We reject the hypothesis that dwarf mangrove leaves have a greater salt gland frequency than those of fringe mangroves. Cytomorphological evidence showed that mangroves survive in the dwarf site by developing xerophytic features such as thicker leaves and cuticles in order to store water and reduce transpiration.

Secondly, we accept the hypothesis that there are distinct ultrastructural differences between fringe and dwarf mangrove leaves. Leaf ultrastructural investigations showed that in comparison to fringe mangroves, chloroplasts and mitochondria swell and lost structural integrity, nuclear membranes appeared fragmented, and mesophyll and epidermal tissue was damaged in dwarf mangroves. Lipid formation and plasmolysis of cell contents from the cell walls commonly featured in cells of dwarf mangrove leaves. Leaf cells in dwarf mangroves developed vesicles and multivesicular structures which enclosed in vacuoles and associated cytoplasmic organelles so as to compartmentalise ions and reduce specific ion toxicity. Salt glands of dwarf mangroves actively excreted or secreted salt exudates to the surface to minimise ion build up in the cells.

However, data also indicates that mangrove sustainability and growth decline, as these plants could not cope under stressful conditions of hypersalinity, low water availability and nutrient limitation (Naidoo, 2006). Lastly, we accept the hypothesis that there are distinct physiological differences between fringe and dwarf mangrove leaves. Physiological analyses showed that dwarf mangrove leaves had lower % relative water content, specific leaf area, chlorophyll content and possessed lower salt gland frequencies than leaves from fringe sites. Na^+ was the predominant ion whilst Mg^{2+} , Ca^{2+} , K^+ and phosphorus concentrations were low in dwarf mangrove leaves compared to those of fringe mangroves. It has been shown that dwarf

mangroves have the ability to develop protective mechanisms during water and salt stress by accumulating higher levels of glycinebetaine in leaves. It is believed that glycinebetaine served as an osmoprotectant, preventing ion toxicity in leaves.

The results of this study suggested that there were distinct differences between leaf ultrastructure, morphology and physiology between fringe and dwarf mangroves. Dwarfing is an adaptation of the plants to cope with the environmental conditions of high salinity, high evaporation and ion imbalance in the soil. Additional research is needed to test and further define these conclusions.

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